HUMAN OVARIAN FOLLICULAR DYNAMICS DURING NATURAL MENSTRUAL CYCLES AND ORAL CONTRACEPTION CYCLES

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by

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ABSTRACT

The objective of the research comprising this thesis was to characterize ovarian follicular development in healthy women of reproductive age undergoing natural menstrual cycles and oral contraception (OC) cycles. We quantified changes in the numbers and diameters of follicles, detected ovulation and assessed changes in the growth and regression of corpora lutea using high-resolution transvaginal ultrasonography. Changes in follicular and luteal development were then correlated with changes in concentrations of reproductively-active hormones and endometrial growth to provide a comprehensive approach to ovarian and uterine function.

We documented, for the first time, that women exhibited waves of antral follicular development during the menstrual cycle. Two and three waves of follicle growth were observed. Major and minor waves of follicle development were characterized. Major waves were those in which a dominant follicle was selected for preferential growth; minor waves were those in which dominance was not manifest. Luteal progesterone production appeared to have a negative effect on the emergence and development of follicle waves in women. The ovarian follicular wave phenomenon has provided a new model for studying the growth and regression of ovarian follicles during the human menstrual cycle. Documentation of ovarian follicular waves in women has implications for the development of new strategies to manipulate ovarian follicular development, in particular hormonal contraceptive regimens and infertility therapies.

We further documented that ovarian follicular development occurred during the compliant use of oral contraception. Follicles developed to ostensibly ovulatory diameters, and either regressed, ovulated or formed follicular cysts under the suppressive effects of OC. The majority of follicles that developed during OC use emerged during the hormone-free interval (HFI). We interpreted our findings to mean that ovarian follicular development during OC use was associated with loss of endocrine suppression during the HFI, rather than user non-compliance as previously speculated. The number and maximum diameter of follicles that developed during OC use were greater in women administered OC containing 20 μg versus 30–35 μg Ethinyl

Estradiol formulations. Our results provided rationale for a reduction or complete elimination of the HFI in OC regimens, and the judicious use of low EE dose OC regimens (i.e., $\leq$ 20 μg EE). Ovarian follicular development and circulating concentrations of estradiol and LH were not suppressed effectively when OC use was initiated at mid to late stages of follicle development (i.e., $\geq$ 10 mm). Our findings demonstrated that dominant follicles secrete estradiol and become increasingly responsive to LH as they acquire functional dominance after becoming physiologically selected for preferential growth during the follicular phase of the menstrual cycle.

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DEDICATION

For my parents Norm and Marilyn

and my husband Mike.

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LIST OF ABBREVIATIONS

ARB = Angela Renee Baerwald

bFGF = basic fibroblast growth factor

°C = degrees celsius

cAMP = cyclic adenosine monophosphate

CL = corpus luteum

COC = cumulus oocyte complex

DNA = deoxyribonucleic acid

DSG = desogestrel

E_2 = estradiol-17β

EC = emergencey contraception

EE = ethinyl estradiol

EGF = epidermal growth factor

FDA = food and drug administration

FSH = follicle stimulating hormone

GH = growth hormone

GnRH = gonadotropin releasing hormone

HAF = hemorrhagic anovulatory follicle

hCG = human chorionic gonadotropin

HFI = hormone free interval

hMG = human menopausal gonadotropin

IGF = insulin-like growth factor

IGFBP = insulin-like growth factor binding protein

IL = interleukin

IOI = interovulatory interval

IWI = interwave interval

LH = luteinizing hormone

LNG = levonorgestrel

LUF = luteinized unruptured follicle

MHz = mega hertz

mIU = milli international units

mL = millileters

mm = millimeters

mRNA = messenger ribonucleic acid

ng = nanogram

NGF = nerve growth factor

NGM = norgestimate

NO = nitric oxide

NPV = numerical pixel value

OC = oral contraception

OMI = oocyte maturation inhibitor

P_4 = progesterone

PA = plasminogen activator

PAF = platelet activating factor

pg = pictogram

pmol = picomole

r = correlation co-efficient

RAP = Roger Allen Pierson

SCF = stem cell factor

SD = standard deviation

SEM = standard error of the mean

TGF = transforming growth factor

USA = United States of America

μm = micrometers

Chapter 1

GENERAL INTRODUCTION

1.1 Human Ovarian Follicular Dynamics

The discovery of the human "ovarian follicle" and "ovulation" in 1672 by the Dutch anatomist Regnier de Graaf (1641-1673) paved the way for a new era of reproductive science and medicine [1]. Although de Graaf initially believed the fluid-filled "follicle" to be the egg, his work led to further investigations by Karl Ernst von Baer in 1827 to describe the follicle-enclosed "ovulum" in mammals [2]. From these early findings, the Latin phrase "Ex ovo omnia" came to exist (translated as 'All organisms begin as eggs')[3]. Extensive attempts have since been made to understand ovarian anatomy and physiology and their impact on female health and reproduction.

The onset of the 21st century and advent of new technologies has resulted in an unprecedented expansion of knowledge in women's health care. Growing global population and productivity have demanded more intense reproductive regulation, culminating in an increased interest in contraceptive methods and paradoxically to an increase in infertility. The quest for insight into female reproduction has become extremely specialized, and we are far from understanding its intricasies. The research contained in this thesis focuses on the development of ovarian follicles during natural menstrual cycles and under the suppressive effects of oral contraceptives. Much of the current knowledge on ovarian follicular development in women has been extrapolated from studies performed in non-human primates, rodents and farm animals. In this review, data on human folliculogenesis is discussed and supplemented with related accounts in animals where necessary.

1.1.1 The Follicular Reserve

In the human ovary, follicular development begins as early as the fourth month of fetal life [4]. It is at this time that the primordial germ cells have migrated from the yolk sac endoderm to the gonadal ridge, undergoing mitotic divisions. Somatic cells (i.e., surface epithelial cells, follicular granulosa and theca cells, interstitial cells, fibroblasts) originating from the primitive gonad surround the oogonia, forming a rudimentary ovarian follicle [5]. Once arriving at the genital ridge, the oogonia enter the first meiotic division and become oocytes. The majority of oocytes become arrested at the dictyate stage of prophase I until puberty. Follicles containing these oocytes constitute the ovarian follicular reserve (total number estimated at approximately 7 million), serving to provide a woman with all of the germ cells for her entire lifetime [4].

Morphologic studies of infant and adult ovaries have identified 3 types of non-growing follicles: i) Primordial follicles, in which the oocyte is surrounded by a single layer of squamous granulosa cells, ii) Intermediary follicles, in which the oocyte is surrounded by a single layer of both squamous and cuboidal granulosa cells, and iii) Primary follicles, in which the oocyte is surrounded by single layer of cuboidal granulosa cells [6]. Primordial and intermediary follicles are the main components of the follicular pool. Primordial follicles are 30 to 60 μm in diameter, while primary follicles are 60 μm. Dictyate stage oocytes measures 9 to 25 μm, and are referred to as primary oocytes [7]. Granulosa cells of the primary follicles synthesize and secrete mucopolysaccharides, giving rise to a translucent halo surrounding the oocyte referred to as the zona pellucida [8]. The zona pellucida most likely originates from the oocyte, granulosa cells, or both [9].

Human follicular growth, in its entirety, begins at the primordial follicle stage and continues for more than 12 menstrual cycles [10]. The 'transition' from the primordial to primary follicle stage in women is an extremely slow process that is not fully understood. There is increasing evidence to suggest that the oocyte may produce factors which stimulate the transition of primordial follicles from the reserve pool to the growth phase [11, 12]. In contrast to the 'transition hypothesis', different types of resting follicles in mammals have been observed at different locations in the ovary (i.e.,

larger follicles occupying the inner zone, and smaller follicles occupying the outer zone). Therefore, it has been speculated that the different types of resting follicles may have distinct embryological origins [13].

1.1.2 Pre-antral Growth Phase

The number of follicles occupying the resting follicular pool is depleted as a woman ages. The depletion of the follicular reserve is due to 2 factors: 1) follicular growth (i.e., folliculogenesis) and 2) regression (i.e. atresia), both of which may occur at any given stage of a woman's life. As follicles enter the growth phase, both the follicle and oocyte enlarge. Follicles leave the resting pool and begin to grow when the germinal vesicle reaches a diameter of 19 μm [6]. The primary oocyte becomes surrounded by increasing layers of cuboidal granulosa cells, forming secondary follicles at a diameter of < 120 μm [7, 10]. Once 3-6 layers of granulosa cells are observed, theca interna and externa layers form and become separated from the granulosa cells by a basal lamina. Secondary follicles become preantral follicles when at least one theca interna cell becomes epitheloid [14]. It is from this stage of development onward that FSH, estrogen, and androgen receptors have been detected in granulosa cells [15]. Differentiation of follicular vascular and lymphatic circulatory systems occur, and thecal cells acquire LH receptors [15, 16]. Mature secondary follicles migrate into the ovarian medulla and grow into pre-antral follicles containing approximately $3\text{-}5x10^3$ granulosa cells, categorized as class 1 follicles [10]. It is believed that that resting primordial follicles are continuously moving to the pre-antral stage [10]. Pre-antral follicular development has been shown to be symmetric between both ovaries [10].

1.1.3 Early Antral Growth Phase

The early antral phase of follicular development describes the conversion of class 1 (pre-antral, 0.1-0.2mm) follicles into class 2 follicles (early-antral, 0.2-0.4 mm) [10]. Early antral follicles have also been referred to as tertiary follicles [8]. Follicles enter class 2 approximately 25 days after entering class 1, between days 11 and 14 of the cycle. Early antral follicles are characterized by the proliferation of $1.5x10^4$ granulosa cells, and the development of a small fluid-filled cavity called the antrum

[10]. Localized formation of lacunae, termed Call Exner Bodies, can be visualized histologically within the granulosa cells. Although their role has not yet been elucidated, lacunae develop in parallel to antrum formation. Antral fluid forms an ultrafiltrate, containing free and protein-bound sex-steroid hormones, plasma and locally derived proteins, proteoglycans, and electrolytes. The establishment and changes in antral fluid hormone concentrations throughout the cycle are not fully understood, although it is believed that some of the steroids are secreted by the follicular cells and transported across the basal lamina into the antrum by diffusion [8] or active transport [17, 18]. Conclusions made from rodent studies have suggested that the antrum forms due to FSH-stimulated estradiol-17ß levels in the late follicular phase [10], at which time the number of follicles with a small antral cavity peaks [14].

1.1.4 Antral Growth Phase

Class 2 follicles undergo a transition to class 3 follicles in the late-luteal to early-follicular phase of the subsequent cycle. Class 3 follicles have been estimated to be 0.4-0.9 mm in diameter with $7.5x10^4$ granulosa cells [10]. Approximately 15 days later, class 3 follicles are converted to class 4 follicles (0.9-2.0mm), which contain $3.7x10^5$ granulosa cells. Follicles then enter class 5 (2-5mm) within the next 10 days in the late luteal phase, and consist of $1.9x10^6$ granulosa cells. These follicles have been referred to by some as 'selectable follicles' [14]. Growth up to the 2-5 mm range has been termed 'basal' or 'tonic' follicular growth [6]. Follicles < 4 mm in diameter can be visualized histologically [14, 19] at any stage of the cycle. Follicular development beyond the 2-5mm stage, characterized by the enlargement of the antrum and differentiation of follicular cells, is referred to as 'terminal' follicular growth [13].

1.1.5 Regulation of basal follicular growth

The stimuli for initiating and maintaining early follicular growth are not fully understood. It is believed that factor(s) may act directly to stimulate follicular growth or transform the granulosa cells to make them more responsive to growth-inducing signal(s). The most probable candidates are the gonadotropins. It has been reported that follicles grew independently of gonadotropins until ~ 0.25 mm, after which time

they required basal FSH and LH to develop an antrum and continue growth to the 2-5mm stage [20-22]. Development beyond 5 mm is believed to require cyclic pituitary activity [21].

In contrast, others reported that development beyond the primary follicle stage, occurring as early as 7 months gestation, required both FSH and LH [22, 23]. The development of more sensitive assays and the finding that FSH receptors are present on granulosa cells of preantral follicles, further supported a role of gonadotropins in pre-antral follicular development [15]. DNA synthesis and cell proliferation in human preantral follicles have been influenced by gonadotropins, especially FSH [24]. It has been postulated, based on rodent data, that FSH levels are low enough during basal growth to inhibit desensitization of adenylate cyclase in granulosa cells of pre-antral follicles. As a result, these follicles are allowed to undergo continuous, undisrupted development throughout the cycle [14]. Follicles undergoing basal growth in vitro produced very low levels of progesterone and androstenedione [24]. However, steroidogenic enzyme activity in human follicles smaller than 2 mm has not yet been reported [25].

It is well-documented that cAMP-mediated signal transduction mechanisms are activated following gonadotropin receptor binding during differentiation and maturation of follicular granulosa and thecal cells [8]. Currently, research is underway to identify the genes that are expressed during the processes of follicular growth and atresia in animal species [26-29].

Oocyte, theca and/or granulosa cell-derived growth factors have been implicated in the regulation of pre-antral and early antral follicular growth. Growth factors are often ubiquitous and may act in paracrine, autocrine, endocrine, juxtacrine, or intracrine manners. Possible growth factor candidates for the regulation of early follicle growth include Insulin-like Growth Factor-I (IGF-I), Insulin-like Growth Factor-II(IGF-II), Insulin-like Growth Factor-Binding Proteins (IGFBPs), Growth Hormone (GH), Epidermal Growth Factor (EGF), Transforming Growth Factor (TGF), Basic Fibroblast Growth Factor (bFGF), Nerve Growth Factor (NGF), inhibin, activin, follistatin (activin binding protein), glycosaminoglycans, oxytocin, substance P, Interleukin-1 (IL-1), Interleukin-6 (IL-6), Tumor Necrosis Factor a (TNFa), interferon, luteinization

inhibitors, gonadotropin binding inhibitors, placental proteins, plasminogen activator, and OMI (oocyte maturation inhibitor) [9, 30].

Most of the literature on growth-factor mediated follicular development is based on data from non-human primates, rodents and domestic animal species. Ovarian growth factor production may be under gonadotropic regulation [31] or growth factors may act to regulate gonadotropin and gonadotropin receptor expression [30]. Mechanisms regulating the gonadotropin receptor and the transducing mechanisms to which they are coupled are not currently understood in primates [14]. It appears that FSH modulates growth factor and growth factor receptor expression and synthesis in pre-antral follicles [24]. Insulin-like Growth Factor 1 may act in synergy with GH to amplify gonadotropin action [16]. It has further been suggested that an inhibitory factor may be acting locally and/or systemically to prevent follicles from leaving the resting pool. Therefore, initiation of follicle growth may be triggered by the disappearance of an inhibitory influence [14]. Epidermal growth factor, TGF or OMI may be potent inhibitors of gonadotropin-supported granulosa cell differentiation [16]. Determination of the regulatory roles of growth factors in initiation and maintenance of basal growth in women awaits further investigation.

1.1.6 Recruitment

The term 'recruitment' has been used frequently in the past 50 years by different researchers to describe different events. To avoid confusion, McGee et al. characterized 2 different types of recruitment in women: 1) initial and 2) cyclical recruitment [32]. The continuous entry of follicles into the growth trajectory from the primordial pool (discussed earlier) described 'initial recruitment'. 'Cyclical recruitment', on the contrary, referred to an FSH-induced growth of a cohort of antral follicles (i.e., the rescue of a cohort of antral follicles from atresia) [33, 34]. The majority of current literature uses the term 'recruitment' synonymously with 'cyclical recruitment'. For the sake of simplicity, we will use the 'recruitment' to describe 'cyclical recruitment' in this review.

Reports on the time of recruitment in women are ambiguous. DiZerega proposed 3 possible modes of recruitment during the primate menstrual cycle: 1) after

the mid-cycle LH-FSH surge, 2) during the late-luteal phase due to elevated FSH levels and 3) after the fall in luteal phase progesterone concentrations at the onset of menses [35]. Hodgen and Hillier stated that recruitment occurred symmetrically (i.e., in both ovaries simultaneously) during cycle days 1-4, at the onset of menses [36, 37]. At present, it is generally accepted that follicles measuring 2-5 mm in diameter are recruited for further growth at the end of each luteal phase [33].

It has been estimated that the cohort of recruited follicles (i.e., 2-5 mm) in women aged 24-33 consists of 3-11 follicles per ovary [34]. This number of follicles contrasted to the total number of antral follicles >1 mm in women which equalled 6-46 per ovary [38]. The recruited cohort represented a group of follicles at a comparable, but not identical, stage of development [13]. It is believed that these follicles left the primordial pool, by chance, around the same period of time several months before [39].

Follicles have been shown to continuously leave the primordial pool from gestational age onward. However, recruitment of follicles to the 2-5 mm stage occurred only after puberty, when circulating gonadotropin levels rose above basal levels after regression of the corpus luteum [22, 40]. Luteectomy studies in monkeys have confirmed the relationship between luteal demise and follicular recruitment. Ablation of the corpus luteum resulted in a prompt decline in serum progesterone levels to early follicular phase levels, recruitment of a new cohort of follicles, and menses 3-4 days later followed by a typical ovulation at mid-cycle [37, 41]. Luteolysis is associated with an increase in LH pulse frequency from <1 per 6 hours during the mid-luteal phase to nearly 4 per 6 hours in the early follicular phase. Increased LH pulse frequency is presumably reflective of a gradual increase in GnRH pulse frequency, resulting in an increase in the concentration of LH and a relatively greater increase in the concentration of FSH [38].

Follicle stimulating hormone is believed to be the primary gonadotropin responsible for follicle recruitment [37]. FSH levels begin to rise 12 days after the LH surge in women [39]. The FSH 'threshold' concept suggests that FSH must be elevated 10-30% above a threshold in order for follicles to escape atresia and continue development [41]. Any further increases are believed to result in excessive stimulation. Fauser and Van Heusden proposed that the duration of the FSH 'window', rather than

the magnitude, is responsible for determining the number of follicles to be recruited. The longer FSH levels are elevated in the early follicular phase, the greater the number of follicles that will continue growth [39]. The FSH threshold is not fixed, but rather is dependent on the developmental stage of the follicle. The FSH threshold therefore changes over time and differs from one individual to the next.

Follicles within the recruited cohort become more responsive to gonadotropins and have been shown to produce androgens (primarily androstenedione). However their ability to express aromatase activity is debated. Some researchers have failed to detect aromatase activity in recruited follicles, presumably due to factors, such as EGF, TGF, and IGFBPs which may act to inhibit aromatization of androgens [10]. In contrast, others suggested that the granulosa cell aromatase system in each follicle is activated as the FSH threshold is surpassed during recruitment [37]. It is currently thought that estrogen is produced by granulosa cells of follicles greater than 1 mm, but remains at low levels (<200 ng/mL) [42] until the mid follicular phase when a dominant follicle is selected for pre-ovulatory growth [14, 39, 43]. It is believed that estradiol may act as a mitogenic factor for the growth of antral follicles [42].

1.1.7 Selection

According to Gougeon's model, which has become one of the most accepted models of folliculogenesis, class 5 follicles recruited in the late luteal phase continue to grow for 5 days and enter class 6 (6-10 mm) in the early to mid follicular phase. Class 6 follicles are estimated to contain $9.4x10^6$ granulosa cells [10]. It is from this cohort of 6-10 mm follicles that a single follicle is chosen for preferential growth and ovulation during a process called 'selection' [40]. The selection process in women contrasts to that in polyovulatory animals in which more than one follicle is selected and ovulates. The follicle that is selected is referred to as the 'dominant' [44] or 'privileged' [10] follicle, while all other follicles in the cohort follow an asymmetric pattern of development and undergo atresia. Atretic follicles have been termed 'ordinary' [10], 'challenger' [45], 'subdominant' [46], or 'subordinate' [47] follicles. For consistency, the terms 'dominant' and 'subordinate' follicles will be used throughout this review.

Goodman described 2 different theories of selection: 1) Stochastic and 2) Deterministic [40]. The Stochastic approach viewed selection as a random event that continued until the number of follicles remaining in the cohort equalled the species-specific ovulatory quota. The Deterministic approach, which is more accepted today, referred to a process by which specific follicles were chosen for further growth by some unknown criteria.

Follicle selection occurred between days 5-8 of the menstrual cycle at a diameter of approximately 10 mm [35, 48]. Physiologic selection of a dominant follicle occurred when FSH levels were declining [35, 48]. Therefore, it was speculated that the dominant follicle continued to grow due to a greater sensitivity to FSH than subordinate follicles [49]. The dominant follicle was reported to contain a greater number of granulosa cells at the time of divergence than subordinate follicles [10]. Dominant follicles may therefore exhibit greater gonadotropin receptor binding. However, in another study, the number of FSH receptors did not change during antral follicle development until at least 12 mm, suggesting that regulatory factors must be involved in increasing the FSH-sensitivity in the follicle which becomes selected [50].

By day 5-8 of the cycle, aromatase activity has been detected in granulosa cells of follicles larger than 6-8 mm, with the dominant follicle producing more estradiol-17ß than other follicles in the cohort [51, 52]. The dominant follicle is therefore said to demonstrate asymmetric ovarian functioning [19, 40, 53]. Estradiol-17ß produced from the dominant follicle has been demonstrated to induce LH and FSH receptor formation on its granulosa cells and stimulate the synthesis of IGF-I from granulosa cells, resulting in increased gonadotropin sensitivity and continued growth [15, 54, 55, 56]. The increase in IGF-I occurred in association with an increase in IGFBP-protease, also known as Pregnancy-associated Plasma Protein-A [55]. It is believed, based on animal studies, that the dominant follicle becomes more responsive to LH during the process of selection [54].

Follicle Stimulating Hormone, LH and Estradiol-17ß secretion in the mid-follicular phase are believed to be involved in oocyte maturation [31, 57]. The diameters of follicles containing healthy oocytes have been shown to positively correlate with antral estradiol levels in the follicular phase of the cycle [58]. However,

no correlation between follicular diameter and estradiol levels was observed in subordinate follicles [43].

Inhibin and activin are 2 peptide hormones produced by the granulosa cells which are involved in the selection process. Two forms of inhibin exist: inhibin A and inhibin B. Three forms of activin exist: activin A, activin B and activin AB. Inhibin A and B both suppress FSH secretion [59]. A rapid rise in inhibin B has been observed during the early folicular phase [60]. Inhibin B and estradiol-17ß, produced from the dominant follicle suppressed FSH secretion and prevented subordinate follicles from undergoing further growth [59]. Inhibin A increased in the latter part of the follicular phase and reached a peak at mid-cycle [61]. Inhibin B aided in selection and growth of the dominant follicle by stimulating thecal cell androgen production (in synergy with LH) thus providing a substrate for granulosa cell estrogen biosynthesis [62]. A precise role of activin in the selection process has not yet been elucidated. However, activin stimulates FSH secretion and inhibits androgen production by theca cells thereby limiting estradiol secretion by the developing follicle [63].

Granulosa cell aromatase expression, increased inhibin production, and a shift from FSH to LH sensitivity have become hallmarks of the selection process. Ablation of the dominant follicle in monkeys [35] and women [64] resulted in a delay of the LH and FSH surges by about 2 weeks. It can therefore be assumed that the dominant follicle had already been selected at the time of cautery, and that no other follicle was competent to accommodate a timely ovulation. The normal 2-week interval to ovulation was interpreted to mean that a whole new group of follicles was recruited after follicle cautery. These studies, which compare to similar studies performed in cows [65], support the notion that a single follicle in women exerts both morphologic and functional dominance once selection has occurred. Hodgen (1982) characterized passive versus active follicular dominance. Active dominance referred to the suppression that the dominant follicle exerted on the maturation of other follicles, while passive dominance suggested that the dominant follicle thrived uniquely, despite the suppressive milieu [44]. A zone, referred to as a 'corona', has been observed ultrasonographically around the dominant follicle. This zone, devoid of visible antral follicles, became more apparent as ovulation approached. Coronas were detected

around dominant follicles only, presumably due to an inhibitory effect of the dominant follicle on subordinates [45].

It is likely that factors, such as IGF, IGFBPs, EGF, TGF, bFGF, Follistatin, cytokines, and/or FSH receptor binding inhibitor, act synergistically with inhibin and/or estrogen to produce a deteriorating endocrine environment in which only a single advantaged follicle is able to develop LH sensitivity and continue to thrive [39, 66, 67]. It has also been postulated that the dominant follicle secretes a substance which may directly inhibit the growth of subordinate follicles. "Selectron" has been postulated as this substance [40, 68]. Although the production of inhibin and estradiol by the dominant follicle and their inhibitory action on FSH secretion are well documented, conclusive in vivo evidence for a distinct role of growth factors in human ovarian physiology is lacking as yet.

Several studies have been designed to determine whether or not the follicle to be selected has an early size advantage over other follicles within the cohort. Gougeon reported that the largest healthy follicle at the beginning of the follicular phase appeared to be the selected follicle [14]. Similar results have been obtained in the bovine and equine species [69, 70]. However, in other studies, the dominant follicle was found to measure 1mm in diameter at the end of the luteal phase, but was not the largest follicle visualized [71]. In comparison, the largest follicle detected ultrasonographically at the beginning of the follicular phase regressed [71], and the dominant follicle became larger than subordinates in the mid-late follicular phase [45].

Numerous studies have been conducted to determine whether the side of selection and ovulation is dependent on the side of the previous ovulation. "Fixing" the side of ovulation in monkeys by removing the ovary ipsilateral or contralateral to the previous ovulation had no effect on new follicular growth [35]. However, elective cautery of the dominant follicle or corpus luteum almost invariably resulted in the next ovulation occurring on the contralateral ovary [35]. In women, 7/8 dominant follicles grew contralaterally to the corpus luteum from the previous ovulation [19]. Fukuda reported that dominant follicles in contralateral ovulation cycles showed higher estradiol/androstenedione ratios than those of ipsilateral cycles, and total pregnancy rates in insemination and IVF cycles were higher in contralateral than ipsilateral

ovulations [73]. Sonographic evaluation of ovulation in both infertile and fertile women, however, did not support these findings [74-76].

1.1.8 Pre-ovulatory follicular growth

The class 6 follicle continues to grow after it is physiologically selected, enters class 7 (10-16 mm) after ~5 days, and then enters class 8 (16-20 mm) after the subsequent 5 days, reaching pre-ovulatory status in the late follicular phase [10]. Bomsel-Helmreich et al. identified 3 types of large follicles prior to ovulation: 1) pre-ovulatory, 2) healthy non-ovulatory, and 3) atretic non-ovulatory follicles [77]. Pre-ovulatory follicles were round with a smooth antral edge and mid-range echogenicity, as determined ultrasonographically [77]. Non-ovulatory follicles were irregular in shape with an indistinct antral edge and mid-range echogenicity [78]. Studies in our laboratory have characterized atretic follicles with having higher echogenicity and thinner walls than healthy, pre-ovualtory follicles [78]. Non-ovulatory follicles < 6 mm have been reported to be androgenic, while those > 6 mm were estrogenic [21]. Preovulatory follicles were estrogenic, but also became progestagenic, prior to ovulation [19, 21].

In the late follicular-early luteal phase, the first subordinate follicle has been reported to occupy the ovary ipsilateral to the dominant follicle [45, 71]. The largest subordinate follicle ipsilateral to the dominant follicle was consistently larger than the largest and second largest subordinate follicles on the ipsilateral and contralateral ovaries [80]. The number of subordinate follicles was greatest in the ipsilateral ovary by the mid-follicular phase, but greatest in the contralateral ovary by the late follicular phase [45]. These findings are inconclusive and indicate the importance of more extensive investigation into the possible paracrine, endocrine, autocrine-mediated regulation of follicular development. There is also evidence that the dominant follicle migrates toward the fimbrial pole of the ovary prior to ovulation [81]. However, ultrasonographic studies have not yet been done which documented this trend.

The growth rate of the dominant follicle in the follicular phase was reported to be quite variable. Schipper et al. reported a mean linear increase in size of the dominant follicle of 1.5 mm per day until 20.5 mm [82]. Dervain reported that the growth rate

increased by 2-3 mm per day during the 3 days prior to ovulation [83]. Renaud et al. described a growth rate of 3.3 mm per day during the 4 days prior to ovulation [84]. In contrast, Macklon and Fauser cited a growth rate of 1.1 mm/day in the 7 days prior to ovulation [20]. Greater growth rates in the dominant versus subordinate follicles prior to ovulation have been reported [80, 81]. In other studies, however, no difference in growth rates between ovulatory and non-ovulatory follicles was detected [78].

Growth of the dominant follicle resulted in a rapid elevation of circulating estradiol-17ß, with pre-ovulatory estradiol levels reaching 200 pg/mL [42]. The dominant follicle was responsible for over 90% of the estrogen production in the pre-ovulatory period [85]. The day before the LH surge, estradiol production from the dominant follicle peaked with a high estradiol/estrone ratio [86]. Estrogen provided positive feedback at the hypothalamus and pituitary to stimulate the release of LH necessary for inducing ovulation.

Plasma progesterone levels also rose in the pre-ovulatory period of the follicular phase, beginning as early as day 10 [19]. The source of this progesterone is somewhat controversial. In some reports, the theca cells were determined to be the source of progesterone prior to the LH surge and the granulosa cells thereafter [87]. Others stated that progesterone was produced initially from the adrenals, and later from the granulosa cells [88]. Pre-ovulatory progesterone has also been shown to be synthesized from stromal interstitial cells, produced from the thecal cells of atretic follicles [89]. Appropriately low levels of progesterone produced by the maturing follicle in the pre-ovulatory phase facilitated the positive feedback action of estrogen, precise synchronization of the mid-cycle LH/FSH surge [88] and luteinization of granulosa cells [77, 79, 80]. An increase in 17α-hydroxprogesterone also occurred prior to the LH surge [90]. 17α-hydroxprogesterone did not appear to contribute to cycle regulation, but rather represented an intermediate product steroid [88]. As LH levels rose in the late follicular phase, the pre-ovulatory follicle shifted from an estrogen-secreting state into a progesterone secreting state [19].

1.1.9 The Role of Growth Factors in the Regulation of Terminal Follicular Growth

It was initially postulated that growth factors had a primary role in regulating basal follicular growth and a lesser role in terminal growth [13]. However, there is increasing evidence to suggest that growth factors may also play a role in terminal follicular development.

Insulin-like growth factors I and II are low molecular weight peptide hormones which mediate the actions of growth hormone (GH) and promote granulosa cell mitosis and differentiation. Although levels of IGF-I and IGF-II have not been shown to differ during the menstrual cycle [91], IGF-I was expressed in thecal cells from small antral follicles (5-7 mm) and IGF-II in granulosa cells from pre-ovulatory follicles [92]. Insulin-like growth factors may amplify the actions of gonadotropins on follicular cells by modulating their signal transduction [62, 92]. Insulin-like growth factor I has been shown to stimulate oocyte maturation, progesterone synthesis, aromatase activity, mRNA expression, and amino acid accumulation in human granulosa cells, while IGF-II increased estradiol and progesterone production. Insulin-like growth factor I, II and IGFBP proteases have been detected in healthy estrogenic follicles, while IGFBPs have been detected in atretic androgenic follicles [91, 93, 94]. Granulosa cells of the dominant follicle have been shown to produce more IGF-II than subordinate follicles [91].

The role of haematopoeitic growth factors, known as cytokines, in follicular development has also emerged. Tumor necrosis factor inhibited FSH-stimulated aromatase activity in rats and may play a role in follicular atresia. However, TNF and IL-1 may also regulate hCG-stimulated progesterone production and FSH-stimulated estradiol production [62]. Tumor necrosis factor and EGF may promote cell division, thecal vascularity, and oocyte maturation, but seemed to have a lesser influence as follicular diameter increased [14]. Most cytokines appeared in the follicular fluid only prior to ovulation [14]. Basic Fibroblast Growth Factor has been shown to promote cell division and angiogenesis, while Nitric Oxide (NO) may be involved in follicular atresia by promoting cytotoxicity and inhibiting steroidogenesis [14, 95]. Vascular endothelial growth factor was found to increase follicular vascularity [96].

Most of the supporting evidence documenting a role of growth factors in regulating terminal follicular growth is derived from animal studies, with limited in vivo human studies. The source and function of these factors in women are not fully understood, and it is very likely that species-specific differences exist in growth factor expression. Further studies in humans are needed before any firm conclusions can be drawn about the growth factor-mediated regulation of follicular growth.

1.1.10 Ovulation

Ovulation is a complex event characterized by a series of morphologic, physiologic, and biochemical changes. Approximately 36 hours after the onset of the LH surge, or 24 hours after its peak, the pre-ovulatory follicle ruptures in the process of ovulation and the oocyte is released into the fimbriated fallopian tube in anticipation of being fertilized [81]. Ovulation has not been reported to occur more than 48 hours after the LH surge [81].

The LH surge sets into motion a proteolytic cascade of events, causing structural changes to take place in the connective tissue of the tunica albuginea and theca externa of the follicle wall as the extracellular matrix and thecal collagen dissociate. Platelet Activating Factor (PAF) stimulated the production of Plasminogen Activator (PA) which catalyzed the conversion of Plasminogen to Plasmin. Plasmin then stimulated the conversion of Procollagenase to Collagenase, which acted as a proteolytic enzyme to break down collagen within the follicular apex [97]. As thecal collagen dissociated, the follicle wall thinned at the apex, focal ischemia ensued, and communication between the granulosa and thecal cells diminished [98]. A role of apoptosis in the cellular breakdown of the follicle wall and subsequent luteinization has been documented [99].

Ovulation has been shown to occur at constant pressure [97]. Luteinizing Hormone induced increased follicular volume, resulting in increased compliance and decreased tensile strength of the follicle wall. Smooth muscular contractions were also observed during follicle rupture. Increased vascular permeability occurred, resulting in vascular leakage and follicular edema [96]. Prior to ovulation, the apical follicle wall thinned and the deep internal wall became thicker, as determined by transvaginal

ultrasonography [27, 100]. A stigma formed at the apex as an outward projection of the follicle wall, and the follicle became flaccid and aspherical [101]. At some point, the follicle wall was no longer capable of containing its follicular fluid and the follicle ruptured, releasing the cumulus oocyte complex (COC) [97]. The event of ovulation began with fluid leakage from the stigma and ended with apposition of the follicle walls. The mean duration of ovulation was 7 minutes (range = 6 seconds to > 18 minutes) [102].

The process of ovulation has been likened to an inflammatory event, due to the acute haemodynamic, cellular and biochemical changes that occurred at the site of follicle rupture [103, 104]. Eicosanoids derived from lipoxygenase and cyclooxygenase are believed to mediate the proteolytic pathway. The renin-angiotensin system, oxygen free radicals, and immune cells may also be involved, although their exact roles are not yet known [97]. It has further been shown that LH tightly regulated proteolysis during ovulation and luteinization by stimulating the coexpression of proteolytic inhibitors [97]. Luteinizing Hormone has been shown to stimulate the production of prostaglandin E_2 and $F_{2\alpha}$, although their role in the ovulatory process is not yet known [23]. Hillier and Tetsuka postulated that glucocorticoids may played an anti-inflammatory role in the ovary, promoting rapid healing of the wound created by follicular rupture [105].

With the onset of the LH surge, the primary oocyte (which has been arrested at the dictyate stage of prophase I) completes the first meiotic division and becomes arrested at metaphase II to become a secondary oocyte [106]. It has been suggested that an inhibitory factor, such as Oocyte Maturation Inhibitor (OMI), may inhibit maturation of the oocyte prior to ovulation. The LH surge may then inhibit the action of OMI, allowing resumption of meiosis [23]. There is also evidence to indicate that the oocyte may produce factors which are involved in resumption of meiosis and cumulus expansion prior to ovulation as well as disassembly of the COC after ovulation [11].

The COC can usually be visualized attached to the follicle wall 2-3 days prior to ovulation, using high-resolution ultrasonography [107, 108]. Follicles in which the COC was not visualized grew to a smaller pre-ovulatory diameter than follicles with an

apparent intact COC [109]. The progesterone-influenced mid-cycle rise in LH is believed to free the oocyte from follicular attachments prior to ovulation [88].

The diameter of the pre-ovulatory follicle just before ovulation is variable, ranging from 15-28 mm [34, 81, 100, 110]. Baird suggested that the size of the pre-ovulatory follicle is species-specific and directly proportional to the species-specific size of the corresponding luteal gland [38].

An association between season and time of ovulation has been documented in women. Ovulation was found to occur primarily in the morning during Spring, and in the evening during Autumn and Winter. From July to February in the Northern Hemisphere, ~90% of women ovulated between 4 and 7 p.m. During spring, 50% of women ovulated between midnight and 11 a.m. [88]. These interesting observations and others have brought forward the concept of seasonality of human reproduction. The concept of seasonal variations in reproductive status has been described in cycling animals [111].

1.1.11 Duration of Human Folliculogenesis

The total duration of folliculogenesis is estimated to be approximately 12 menstrual cycles [10]. The time period for a primordial follicle to grow to a primary follicle is not known but is estimated to occupy >150 days [10]. Likewise, the transition from a primary to secondary follicle has been shown to take > 120 days, a secondary to a selectable follicle takes 71 days, and a selectable to pre-ovulatory follicle takes 14 days [32]. According to Gougeon's classification, the progression from a class 1 (pre-antral) to class 8 (pre-ovulatory) follicle required 85 days [10]. The most extensively studied period of folliculogenesis (i.e., from recruitment to ovulation) occurs within the last 2 weeks of a follicle's lifetime.

1.1.12 Follicular Atresia

Within the first few months of fetal life, the ovaries contain the maximum number of follicles to supply a woman for her entire reproductive lifetime (i.e., approximately 7 million) [4]. The size of the follicular pool continuously decreases so that the total number of resting follicles at birth is approximately 2 million [112]. By

the time a woman has reached menarche, approximately 400,000 - 500,000 follicles are present [4], less than 10% of the original follicular reserve [20]. By menopause, occurring at an average age of 52, the follicular reserve diminishes to only a few thousand or hundreds of follicles [113, 114]. Total exhaustion of the follicular pool in women has been predicted to occur at approximately 74 years of age [114].

Studies have demonstrated that the decrease in the follicular reserve with age occurred as a result of 2 basic processes: 1) Follicular growth (discussed earlier) and 2) Follicular atresia. Follicular atresia is defined as a degenerative process in which all follicular components (including the oocyte) undergo cytolysis, and follicular tissue is replaced by fibrous tissue [22].

Gougeon suggested that the depletion of the follicular pool in younger women was caused mainly by atresia, while that occurring in older women results from entrance of follicles into the growth phase. Rising FSH levels occurring with the onset of menopause, may increase the rate of recruitment, and from this cohort of recruited follicles, it is presumed that the majority will undergo atresia [113]. It has recently been shown that as the number of primordial follicles declines with age, so does the meiotic competence of oocytes [115].

Approximately 99.9% of all ovarian follicles succumb to atresia at some point during their development [116]. Therefore, less than 1% of ovarian follicles (approximately 400 follicles) will ovulate in a woman's lifetime [37]. Billig et al. discussed 5 stages of degenerative ovarian cell loss (i.e., attrition) during ovarian development: 1) During migration of the primordial germ cells from the yolk sac to the genital ridge, 2) Coincident with their entry into meiosis before follicle formation, 3) At the penultimate stage of development (i.e. recruitment) when early antral follicles either differentiate or regress, 4) At the pre-ovulatory stage, if ovulatory signals are absent (or aberrant) and 5). During luteolysis [117]. At each stage of growth, a follicle requires an optimal set of stimulatory factors. A decline in the concentrations of stimulatory factors below threshold levels or overexpression of inhibitory factors may result in follicular degeneration [13].

It has been estimated that 16% of follicles < 1.0 mm, 34% of follicles < 2.0 mm, 62% of follicles < 4.0 mm and 48% of follicles < 6.0 mm are atretic [19].

McNatty et al. reported that 92% of antral follicles in the human ovary less than 10 mm were atretic, whereas more than 50% of follicles greater than 10 mm were healthy, based on histologic examination of the ovaries [110]. These data suggest that most antral follicles underwent atresia during early antral development.

The first signs of atresia in primary follicles have been reported to occur in the cortico-medullary junction of the ovary [22]. Early signs of atresia include the appearance of macrophages around the follicle, an increase in the granulosa cell and cytoplasmic nuclear pycnotic indices, a decrease in the number of granulosa cells by 50%, as well as increased numbers of mutivesicular bodies and lipid droplets, dilation of smooth endoplasmic reticulum and golgi apparatus, and irregular mitochondria in granulosa cells [56, 118-120]. Follicular deterioration at more advanced stages of atresia in several mammalian species has been characterized by the detachment of the granulosa cell layer from the basal lamina, fragmentation of the basal lamina, lipid and vacuole accumulation, cellular debris in the antrum, reduced granulosa cell protein and DNA synthesis, oocyte fragmentation, disruption of the oocyte-cumulus connection, rupture of granulosa mitochondrial and oocyte nuclear membranes, and thecal cell hypertrophy/degeneration [117, 120]. In several mammalian species, the granulosa cells of atretic follicles regressed, but the thecal cells remained to constitute masses of interstitial gland tissue [121]. Interstitial cells present in the ovarian stroma synthesized C19 steroid hormones [89].

Animal studies have revealed that atresia is an apoptotic process whereby affected cells exhibited reduced cytoplasmic volume, nuclear and cytoplasmic fragmentation resulting from DNA cleavage, and ultimately phagocytosis by macrophages [99, 122, 123]. Apoptosis is derived from the ancient Greeks, meaning to "fall off, like leaves from a tree" [88]. Apoptosis differs from necrosis, in which random pathologic cell death occurs [124]. Apoptosis does not elicit an immune response at the site of cell loss, as does necrosis [124]. Apoptosis is a genetically-programmed physiologic cell death process, believed to be regulated by hormones, growth factors, and cytokines [122] of both intracellular and extracellular origin [13]. Atresia has been characterized by high androgen levels, low aromatase activity, low estradiol levels, a decreased response of granulosa cells to FSH, and low inhibin levels

[77, 125]. Byskov stated that the general process of atresia did not appear to be temporally related to marked changes in serum gonadotropins because atresia could occur at any stage of the reproductive cycle [126]. However, it has been shown both in vivo and in vitro in animals that FSH and/or LH are responsible for inhibiting apoptosis in granulosa cells of developing follicles [124]. Therefore, factors which act to inhibit the gonadotrophic response are most-likely involved in the process of atresia [37, 127].

It is believed that, before the onset of puberty, basal FSH levels allow follicular development to progress to the pre-antral or early antral stage. Recruitment does not yet occur, and therefore all follicles undergo atresia. With puberty, FSH levels rise beyond a critical 'threshold' and follicular recruitment ensues. The recruited follicles continue growth, but only one follicle (the follicle with the greatest FSH sensitivity) is selected for pre-ovulatory growth. All other follicles within the cohort undergo atresia [37]. The secretion of inhibin from the granulosa cells of the dominant follicle suppresses FSH secretion by the pituitary, rendering subordinate follicles atretic [88].

Human atretic follicles exhibited decreased estrogen production and increased androgen production [117]. The decreased estrogen in atretic follicles was characteristic of several mammalian species (human, ovine, porcine, rat, hamster). However, alterations in androgens levels appeared to be species-specific, as rat and hamster atretic follicles exhibited decreased androgen levels [117]. Gougeon however, noted that healthy antral follicles < 8 mm possessed low aromatase activity and therefore a high androgen/estrogen ratio similar to that of early atretic follicles of similar sizes [14]. After reaching a diameter of 8 mm, the androgen/estrogen ratio in healthy follicles became less than that observed in atretic follicles [14]. It may therefore be more appropriate to characterize follicular viability based on androgen/estrogen ratios only after follicles reach 8mm.

Administration of EGF, TGF, bFGF, GH, IGF-1, IL-1, NO, activin, FSH and hCG in vitro have been shown to inhibit spontaneous apoptotic DNA cleavage in cultured animal granulosa cells [117, 128]. Inhibition of atresia in preovulatory follicles may occur due to paracrine or autocrine actions of growth factors as well as gonadotropin binding to granulosa cells. It has been reported that IGFBPs markedly increase during atresia, acting to sequester IGFs and reduce the gonadotropic response

[92]. Reactive oxygen species, IL-6, and cytoplasmic proteases (eg. Interleukin Converting Enzyme, Cysteine-protease-P32) have been shown to induce apoptosis in cultured rat granulosa cells [124, 128]. Stem Cell Factor (SCF) and cytokines may prevent apoptosis, although they most likely work in conjunction with additional growth factors [117, 124]. A role of GnRH and/or angiotensinogen in the process of atresia has been suggested, however no definitive evidence yet exists [14]. It is also important to recognize that specific developmental stages of follicle cells determined their susceptibility to survival or apoptotic factors [117]. For example, GH-induced production of IGF-1 suppressed apoptosis in preovulatory, but not in early antral follicles [128]. As during basal and terminal follicular growth, the precise roles of growth factors in human follicular atresia remain to be elucidated.

1.1.13 A Wave Model for Folliculogenesis

The first conclusive evidence that follicles, ranging from the primordial to early growing stage, grew in a cyclic wave-like fashion in women was reported in the early 1950's when Block reported 2 periods of increased follicular growth during the 'sexual cycle' [129]. The first wave of development occurred early in the cycle under the influence of FSH. From this wave, an ovulatory follicle developed. The second wave of development occurred in the early luteal phase. Follicles that grew > 5 mm within the second wave in the mid-late luteal phase were believed to be atretic. More follicles were seen to grow during the post-ovulatory phase; however, follicles grew to a larger diameter in the pre-ovulatory phase. Similarly, Hackeloer and Robinson reported 2 waves of follicular development in 2 women with 30-35 day cycles [107]. In both cases, 1 wave occurred in the follicular phase and the second wave in the luteal phase. The follicular phase was long (>19 days), the luteal phase was short (>10 days), and follicular growth was associated with a rise in estradiol. In one woman, the first follicle reached 16 mm and then became atretic while the second follicle reached 20 mm and ovulated [107]. Similar findings have been reported by Dervain et al. and Gore et al. [46, 83]. In contrast to waves of antral follicle growth, follicular waves in women have also been defined as the entry of pre-antral resting follicles into the growing phase in a continuous manner throughout the menstrual cycle [10]. According to this theory, each

of the follicles observed when sampling one ovary at any given time during the cycle belongs to a different wave of follicular growth.

An increase in the number of ultrasonographically-detected follicles in both the early follicular and luteal phases substantiated a biphasic pattern of follicular development [78]. Furthermore, endocrinologic evaluation of the human menstrual cycle has revealed a biphasic pattern of reproductively active hormones. Luteinizing hormone pulse frequency rose in the early luteal phase (~20 pulses per day) and early follicular phase (~20 pulses per day), compared to the mid-luteal phase (~ 5 pulses per day) [38, 130]. In addition, activin and inhibin were shown to be secreted in a biphasic pattern during the cycle [131]. Activin A was higher at mid-cycle and in the late luteal/early follicular phase with nadirs in both mid-follicular and mid-luteal phases. The opposite trend was documented for inhibin.

Contrary to the studies in which non-random changes in follicular and endocrine endpoints were described, a wave theory of antral follicle development has yet to be clearly documented or defined in women. It has become generally accepted that a cohort of follicles are recruited to grow in the late luteal phase of the menstrual cycle, a single follicle is selected for preferential growth the mid-follicular phase, with ovulation at mid-cycle and limited follicle development during the luteal phase [6, 32, 71, 115]. Follicular development to an ostensibly ovulatory diameter in the luteal phase, as previously described, appeared to represent an abnormal reproductive event. In contrast to a wave pattern of follicle development, some researchers have suggested that a single follicle grew by chance during a hormonally-privileged period of the cycle in women [33]. According to the latter theory, referred to as the "Propitious Moment Theory" [132], antral follicles were recruited and grew continuously until conditions were right for a gonadotropin surge, which stimulated ovulation of the follicles that just happened to be mature at exactly the right point in the cycle [133].

Late antral follicle development in the luteal phase has been thought not to occur during the normal menstrual cycle due to an inhibitory effect of luteal progesterone, estradiol and inhibin on FSH and LH secretion [19, 49, 86, 134-137]. The inhibitory effect of luteal progesterone on follicular development was substantiated by the finding that luteectomy followed by progesterone supplementation in monkeys did not allow

follicular development to occur. Once progesterone implants were removed, a new cohort of follicles was recruited and a normal ovulation occurred [35]. Enucleation of the CL in women resulted in a rise in FSH and LH, and the emergence of a dominant follicle. Seventeen days after enucleation, LH, FSH, and progesterone rose and ovulation occurred [138]. The negative regulatory effect of the CL on follicular growth was further supported after documenting lesser follicular diameter and estradiol levels in follicles on the ovary ipsilateral to the previous ovulation [139].

Follicles > 10 mm have been documented during the late luteal phase [57]. However, the evaluation of oocyte viability and granulosa cell recovery in luteal phase follicles 1-15 mm in diameter determined that these follicles were atretic [42]. The androstenedione/estradiol ratios in these luteal phase follicles were found to compare to those observed in follicles < 8 mm in the follicular phase [110]. Follicles during the mid to late luteal phase contained high levels of aromatizable androgen (400-2000 ng/mL) and low estradiol levels (< 200 ng/mL) [42]. These follicles exhibited low levels of aromatase, and could not catalyze the conversion of androgens into estrogens until the terminal stages of luteal regression [49, 71].

Waves of follicular development have been precisely characterized during the estrous cycle of domestic animal species (eg. bovine, equine, ovine) [140-143]. Follicle waves in domestic animals were defined as the synchronous growth of a group of follicles, which occurred at regular intervals during the estrous cycle [141]. Greater than 95% of bovine estrous cycles were composed of either 2 or 3 follicular waves [47]. Animals with 2 waves of follicular activity exhibited an interovulatory interval (IOI) of 20 days, while those with 3 waves exhibited an IOI of 23 days [144]. The longer IOI in 3 wave animals was attributed to a longer luteal phase (i.e. longer lifespan of the CL) and an unchanged follicular phase [144]. The reproducibility of wave patterns in the same cow over many cycles has not been investigated [47]. However, it has been shown that lactational status and energy balance influenced patterns of folliculogenesis [145].

The emergence of a group of follicles occurred at regular intervals throughout the estrous cycle and was preceded by a rise in FSH [65, 146]. The final wave of the bovine estrous cycle was ovulatory, while all previous waves were anovulatory [144]. The number of follicles selected in ovulatory waves was species-specific. Furthermore,

major versus minor waves of follicular development have been characterized during the equine estrous cycle [142]. Major waves were defined as those in which a dominant follicle was selected for preferential growth over subordinate follicles [142]. Major waves were then sub-grouped into primary or secondary waves. Primary waves emerged during diestrus and gave rise to an estrous ovulation. Secondary waves originated during estrus or early diestrus and gave rise to a dominant anovulatory follicle, hemorrhagic follicle or a diestrus ovulation [142]. Waves in which selection did not occur were called minor waves [142].

1.1.14 Summary

We have come a long way in our understanding of the ovary and ovarian follicle over the past 300 years. However, there is still much to learn about human ovarian follicular dynamics during the menstrual cycle. Research performed to date has failed to provide frequent, serial profiles of ovarian follicular development and reproductively active hormones over the menstrual cycle. It appears that follicle development in women may occur in a wave-like manner, as previously documented in domestic animals. This notion was based on early documentation of non-random changes in follicle number, follicle diameter and endocrine levels during the cycle, as well as clinical observations of large antral follicles during the luteal phase in healthy, ovulating women of reproductive age. Waves of ovarian follicle development in women have been mentioned occasionally in the literature. However, follicle 'waves' were not clearly defined or explained. A wave model of antral follicle development during the human menstrual cycle has not been proposed as a normal reproductive phenomenon. Continued research efforts are required to determine whether waves of ovarian follicular development occur during the menstrual cycle. We anticipate that research in this area will increase our understanding of the basic biologic and physiologic processes underlying female reproduction, and have implications for the design of more safe and efficacious hormonal contraceptive and infertility therapies.

1.1.15 References

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1.2 Ovarian Follicular Development During the Use of Oral Contraception

1.2.1 Oral Contraception Composition and Use

The development of oral contraception in 1960 [1] dramatically changed the way that couples world-wide viewed family planning. Oral contraceptives (OC) are among the most widely used and studied pharmaceuticals. Combined OC preparations contain an orally-active exogenous progestin and estrogen. Supraphysiologic levels of estrogen and progestin have been shown to provide a negative feedback effect on the hypothalamo-pituitary axis [2]. Reductions in endogenous GnRH, FSH and LH are believed to suppress ovarian follicular development, with the ultimate goal of preventing ovulation and subsequent conception. The progestin component of OC is believed to inhibit the LH surge and ovulation [3-5]. The exact mechanism of action of the estrogen component has not yet been elucidated. However, in primates, estrogens have been shown to inhibit the growth of pre-antral and medium-sized antral follicles [6, 7], presumably through inhibition of FSH secretion. Estrogen is also required to provide satisfactory bleeding patterns in women during regular OC use. It is further believed that OC elicit secondary inhibitory effects on endometrial development, cervical mucous viscosity, cervical dilation, and oviductal motility [8].

The first OC approved for use, Enovid (Searle), contained 9.85 mg of norethynodrel plus 150 μg of mestranol. A multitude of new hormonal contraceptive regimens have since been developed. Concerns about estrogen-related thromboses led to a marked reduction in the estrogen dose from 150 μg to 30 μg, and a switch from the use of mestranol to ethinyl estradiol (EE), a metabolite of mestranol [9]. More recent OC formulations contain EE doses as low as 20 μg, and clinical trials are underway to determine the safety and efficacy of formulations containing 15 μg EE. The development of 3rd and 4th generation progestins (eg. levonorgestrel, deosgestrel, gestodene and norgestimate) has also resulted in better tolerated and more effective OC regimens [8].

Most combined OC formulations consist of 21 days of dosing pills followed by a 7 day hormone-free interval (HFI). A withdrawal menstrual bleed normally occurs during the HFI. Oral contraceptive use is typically initiated on day 1 of menses or on the first Sunday following menses (i.e., to avoid menses on the weekend). The first OC

regimens used were monophasic, in that the same doses of estrogen and progestin were administered throughout the cycle [1]. Biphasic [10] and triphasic [11, 12] regimens were later developed by changing the individual doses of estrogen and progestin during the cycle. Continuous OC regimens are also currently used, in which women take OC continuously for months at a time and discontinue for a withdrawal bleed only a few times a year if they choose [13, 14].

1.2.2 Ovarian Follicular Development and Ovulation during Combined OC Use

Ovarian follicular development is not completely inhibited during standard use of combined OC [15]. The degree of follicular activity that occurs during OC use depends on the type and dose of steroids used, the administration regimen, user compliance and the individual responsiveness of the woman taking the hormones [16].

Twenty-six studies were found in which pituitary-ovarian activity was evaluated in women using OC containing 20-40 μg EE and various progestins. In 17 of the 26 studies, the development of follicles to diameters of $\geq$ 10 mm was detected using ultrasonography [17-33]. Follicle growth $\geq$ 10 mm is of importance because it is at a diameter of approximately 10 mm that follicles become physiologically selected for preferential growth and ovulation during natural menstrual cycles [34]. Selected dominant follicles are those which have acquired morphologic and functional dominance over other follicles of the cohort, and therefore have the greatest potential to ovulate [35].

In 8 of the 26 studies, ovulation was detected by endocrine assessment with or without ultrasonographic visualization of follicle growth and luteal formation [19, 24, 27, 28, 36-39]. In 16/26 studies, ovulations were not detected during OC use [17, 18, 20, 21, 23, 25, 26, 30-33, 40-44]. In the remaining 2 studies, 2 pregnancies were reported despite failure to detect ovulation [22, 29]. It is not currently known why some follicles ovulate during OC use while other follicles do not. It has been speculated that ovulation of follicles that develop during OC use is prevented by inhibition of the LH surge [45]. However, research to confirm this concept is lacking. In many cases, measurement of serum LH and/or progesterone were used solely as determinants of ovulation. In other studies, infrequent ultrasonographic examinations, with or without

endocrine evaluation, were used to detect ovulation. Considering the variability in serum sex steroid levels and day of ovulation during the menstrual cycle [46, 47], it is plausible that accurate detection of ovulation may have required more frequent and comprehensive ultrasonographic and endocrinologic evaluation.

The majority of dominant follicles reported during OC use failed to ovulate, but rather regressed [13, 14, 17, 18, 20, 21, 23, 25, 26, 30-33, 40-44]. However, follicles have developed to ostensibly ovulatory diameters, in association with pre-ovulatory levels of endogenous estradiol during OC use [18, 27, 40, 48]. Large antral follicles that developed under the suppressive effects of OC use appeared ultrasonographically indistinguishable from pre-ovulatory follicles observed during spontaneous menstrual cycles [18]. The ultrasonographic and endocrinologic similarities between large antral follicles that develop during natural menstrual cycles and OC cycles supports the notion that follicles which develop to pre-ovulatory diameters during OC use retain their ovulatory potential.

1.2.3 Follicle Development and Steroid Dose

It has been reported that the extent of pituitary-ovarian suppression during combined OC use appears to be related to the dose of EE, rather than the type and dose of progestin [49-51]. The maximum diameter of detected follicles and number of follicles observed was greater in women taking low EE dose OC regimens (i.e., 20 μg) compared to moderate EE dose regimens (i.e., 30-35 μg)[16, 29]. In addition, 20 versus 30 μg EE formulations have been associated with greater serum FSH and LH levels [52]. Studies designed to ultrasonographically and endocrinologically evaluate the effect of different progestins on ovarian function, while properly controlling for the estrogen component, however, are lacking and emphasize the need for continued research.

1.2.4 Follicle Cysts and OC Use

Follicle cysts (i.e., 'functional ovarian cysts', 'luteinized unruptured follicles' and 'enlarged follicles') have been documented during OC use [24-27, 31, 52, 53]. The definition of follicle cysts varies among reports. Functional ovarian cysts have been

described as non-pathologic follicular cysts (i.e., anovulatory), corpus luteum cysts or other unspecified ovarian cysts measuring greater than 20 mm in diameter, as detected by ultrasonographic or surgical examination [53]. Functional ovarian cysts have also been described as follicles that developed beyond 30 mm in diameter, failed to ovulate and persisted for more than 2 cycles [26]. The latter definition of functional ovarian cysts contrasts to enlarged follicles which were described as follicles that developed beyond 30 mm in diameter but failed to persist for more than 2 cycles [26]. Luteinized unruptured follicles (LUF) have been defined as follicles which reached pre-ovulatory diameters, failed to ovulate and became luteinized, as determined ultrasonographically [54]. Follicle cysts are sometimes referred to as 'follicle-like structures' in the literature [25, 27]. Follicle cysts usually regress within days or weeks of their development [54, 55]. An increased incidence of functional ovarian cysts has been documented in women taking progesterone-only OC [56, 57]. There is also evidence to indicate that women administered multiphasic and low-dose monophasic combined OC may be at greater risk of developing follicle cysts compared to women taking moderate-dose monophasic OC [26, 52]. The significance of follicle cysts and the mechanisms underlying their development during combined OC use have not been fully elucidated.

1.2.5 Follicle Development and Administration Schemes

The day of the cycle on which OC use is initiated influences the risk of follicle development and ovulation. Delaying the initiation of OC use may fail to inhibit endogenous FSH, LH and estradiol concentrations during the early follicular phase and thus increase the likelihood that a dominant follicle will develop. This concept is supported by the finding that women who initiated OC use on day 5 of the menstrual cycle developed more dominant follicles, in association with higher serum estradiol and gonadotropin concentrations, than women who started OC on day 1 [18]. Follicles $\geq$ 10 mm have been reported in the first 7 days of spontaneous menstrual cycles [34]. Therefore, women who use "Sunday Start regimens", in which OC use may be prolonged for up to 7 days following menses, may be at an increased risk of developing follicles and ovulating [58-60]. The risk of ovulation and conception after a delayed initiation scheme, however, has yet to be determined.

1.2.6 Follicle Development during the Hormone-Free Interval

The decision to implement a 7 day Hormone-Free Interval (HFI) during the 28 day OC cycle was made arbitrarily in the 1960s, so that menstrual bleeding during OC use would mimic that observed during natural menstrual cycles. Several years later, researchers have expressed concern over the degree of follicle development that has been observed during the HFI, particularly with low EE dose formulations [15]. Elevated concerns about follicle development during OC use were the result of advancements in our ability to monitor ovarian function using high-resolution transvaginal ultrasonography.

Dominant follicles $\geq$ 10 mm have been ultrasonographically detected during the HFI [45, 51, 61-63]. Similarly, endogenous FSH and estradiol levels at the end of the HFI have been reported to rise to levels which compared to those observed during the early follicular phase of the natural menstrual cycle [18, 45, 50, 64]. However, resumption of OC at the end of the HFI decreased FSH levels irrespective of the presence of dominant follicles [45, 51]. If no dominant follicles developed during the HFI, follicular suppression was maintained. If a dominant follicle developed during the HFI, follicle growth continued despite declining FSH concentrations [45]. Of the 5 studies in which follicle development during the HFI was observed, ovulation was detected in only 1 study [63].

Shortening the HFI from 7 days to 3 or 4 days provided a greater suppressive effect on ovarian follicular development [63, 67]. A new OC regimen, in which unopposed EE is administered for the last 5 days of the 7 day HFI, has also been associated with more effective suppression of ovarian follicular activity than a standard monophasic regimen [63]. The results of these studies advocate for future research to evaluate ovarian follicular development and ovulation after reducing or eliminating the HFI in OC regimens.

1.2.7 Follicle Development following Missed Doses

The effect of missed OC doses on pituitary-ovarian activity is related to the number of pills missed and the time during the cycle when pills are missed. A greater number of missed doses and number of consecutive days of missed doses are associated

with an increased risk of follicle development and ovulation [15, 67-72]. It has also been postulated that the risk of follicular development and ovulation following missed doses is greater in women taking low EE dose formulations (i.e., $\leq$ 20 μg EE formulations) compared to higher doses of EE [15].

The risk of contraceptive failure is greatest when the first and/or last dosing pills are missed and the HFI is extended [20, 62, 73-75]. In such cases, there is a prolonged period of time during which follicle growth is not suppressed. Follicle growth to a potentially ovulatory diameter and increased estradiol levels were observed when the HFI was extended from 1 to 11 days [20, 62, 73-75]. Ovulation following the extension of the HFI was reported in 3/5 studies [20, 74, 75]. In a separate study, human Chorionic Gonadotropin (hCG) induced ovulation of follicles that reached 18 mm after extension of the HFI [76]. These data support the notion that follicles which develop following an extended HFI have the potential for ovulation.

1.2.8 Suppression of Follicle Development over Subsequent Cycles

The consistency of ovarian suppression over the course of several cycles of OC use is not fully understood. The growth of follicles $\geq$ 10 mm in the first cycle of combined OC has been shown to be greatest in the first week [18]. Greater ovarian suppression has been reported in the first treatment cycle as compared to subsequent cycles [16, 21, 25]. In another study, however, greater suppression occurred in the second and third cycles of OC use compared to the first cycle [20]. The time when doses are missed may further influence the timing of follicle development, especially when missed doses extend the HFI [20, 62, 73-75]. Inconsistencies regarding the degree of ovarian and uterine suppression during OC cycles emphasize the need for further investigation.

1.2.9 Summary

Alterations in the composition of OC over the past 40 years have been made in attempt to reduce adverse effects and improve patient compliance while maintaining contraceptive efficacy. However, there is evidence to indicate that reducing the estrogen dose to minimize adverse effects may have compromised the degree of

hypothalamo-pituitary-ovarian suppression, in particularly during the HFI or following missed doses. It is not currently known why some follicles ovulate during OC use while others regress or form anovulatory follicle cysts. Limited studies have been performed to serially evaluate ovarian follicular development in women taking OC. Studies are required to track the daily growth, regression and ovulation of follicles that develop, using ultrasonographic and endocrinologic evaluation of ovarian status. This information would allow the development of more efficacious contraceptive formulations. In addition, this knowledge would provide insight into the mechanisms of action underlying OC as well as newly-developed hormonal contraceptive devices, such as intramuscular injections, subdermal implants, intrauterine delivery systems, vaginal rings, transdermal patches and emergence contraceptive regimens.

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1.3 Transvaginal Ultrasonographic Imaging of the Ovaries

1.3.1 Overview

The development of transvaginal ultrasonography in the mid-1980s profoundly changed our ability to image the ovaries. Transvaginal ultrasonographic imaging is based upon the ability of different tissues to reflect high frequency sound waves [1, 2]. A piezoelectric transducer emits an acoustic pressure wave which is transmitted into the adjacent tissues. The characteristics of the tissue interfaces determine what proportion of the sound wave will be reflected. The reflected portion of the wave is represented on the ultrasound image by shades of gray, extending from black to white. Liquids (e.g. follicular fluid) are said to be anechoic because they do not reflect sound waves and appear black on the monitor. Dense tissues (e.g. cervix) reflect much of the sound wave, appear bright on the monitor and are therefore hyperechoic. Other tissues are represented by various shades of grey, depending on their echogenicity (i.e., ability to reflect sound waves). The portion of the wave that is not reflected is either refracted, scattered or absorbed in the tissue.

The reflected ultrasound wave is received by the transducer and converted into electrical energy. Each electrical signal is amplified and then stored as binary digits in a digital scan converter. An electron beam is displayed as scanning and raster lines on a monitor. Each point of intersection of a scanning and raster line represents a picture element, or pixel. The location of each tissue reflector corresponds to the location of the stored signal in the scan converter, which in turn, corresponds to each pixel. The images of many conventional ultrasound machines are composed of 480 x 640 pixels. The brightness of each pixel is determined by the amplitude of each individual echo signal. The ultrasound image is made up of a number of pixels, each one represented by one of 256 shades of grey. Several beams of sound waves are reflected and processed to comprise the image. Beams are focused to increase the resolution of the image and optimize viewing.

The most common reason for performing transvaginal ultrasonography of the ovaries is to monitor ovarian follicular maturation and ovulation in women during natural menstrual cycles and those undergoing controlled ovarian hyperstimulation and/or ovulation induction prior to insemination or oocyte retrieval for the treatment of

infertility [2-4]. Ultrasonography has also become an essential tool for the diagnosis of ovarian pathology and disease [2, 3]. The ovaries are easily detected in women of reproductive age using real-time, B-mode (i.e., brightness mode) transvaginal ultrasonography. The ovaries are almond-shaped structures that are usually located lateral to the uterus, posterior to the broad ligament, anterior to the iliac vessels and ureters, and inferior to the fallopian tubes [5]. At times, the ovaries may be found in the anterior or posterior cul-de-sacs or other locations within the pelvis. The presence of pelvic fluid in the cul-de-sac may help to delineate the ovaries [6]. Normal, healthy ovaries measure approximately 3-4 cm long, 2 cm wide and 1 cm in the anterior-posterior dimension [5]. Ovarian blood supply is provided by the adnexal branch of the uterine artery and an ovarian branch that passes through the infundibulopelvic ligament [5]. Ovarian vasculature can be easily detected using Color and Power Doppler imaging techniques [7, 8].

Ovaries have a coarse, low-level echo pattern interrupted with anechoic (i.e., dark) areas that represent developing follicles, functional cysts or corpora lutea [5]. Ovarian follicles are visualized, using 2D ultrasonography, as circular hypoechoic (i.e., dark) structures surrounded by a thin more hyperechoic (i.e., brighter) follicle wall [5]. Follicles as small as 2 mm in diameter can be detected throughout the menstrual cycle [3]. A mature follicle generally measures 20-24 mm in diameter [5]. The appearance of a subtle double contour inside the follicle wall is indicative of thecal and granulosa cell differentiation in the pre-ovulatory follicle [9]. Vascularization of the follicle thecal cell layer increases as the follicle develops and can be visualized using Doppler techniques [5]. The cumulus-oophorus can be seen in 60-80% of follicles 12-24 hours before ovulation [10, 11]. The follicle becomes aspherical in shape and develops a stigma at the follicular apex immediately prior to ovulation [11]. The follicle wall thins at the apical border and ruptures in the process of ovulation [11].

The development of the corpus luteum (CL) after ovulation can also be visualized using high-resolution transvaginal ultrasonography [12]. The walls of the evacuated follicle become thicker and more highly-vascularized during luteinization [12]. The outer and inner surfaces of the CL are usually folded and occupy most of the tissues identifiable as the CL. Luteal tissue is generally of mid-range echogenicity [13].

Hemorrhage into the lumen of CL occurs in approximately 60% of women after ovulation, forming a corpus hemorrhagicum [14]. Luteal tissue area and vascularity reach maximal levels 6-7 days after ovulation, after which time luteal regression occurs in association with decreased luteal tissue area and vascularity [13]. The cells of the regressing CL degenerate into an amorphous hyaline mass held together by strands of connective tissue, referred to as the corpus albicans [12]. Corpora albicans are visualized as hyperechoic masses of scar tissue that eventually regress completely over the course of the next few menstrual cycles [12].

Failure to ovulate can result in the formation of ovarian cysts, such as anovulatory follicular cysts, luteinized unruptured follicles (LUF) or hemorrhagic anovulatory follicles (HAF) [15]. Formation of follicle cysts has been associated with infertility. However, follicular cysts have also been reported in healthy women of reproductive age [3]. The incidence of cystic follicles in women during natural menstrual cycles is not currently known. However, it has been estimated that 22% of women undergoing ovulation induction with human Menopausal Gonadotropin (hMG) and hCG fail to ovulate and develop follicular cysts [16]. Anovulatory follicular cysts are visualized ultrasonographically as aspherical in shape, with thin hyperechoic (i.e., bright) walls. The integrity of the follicular wall is often compromised. Echoic structures have been seen protruding into the follicular antrum and floating freely in the follicular fluid (believed to represent cellular sloughing of the follicle wall) [15]. Hemorrhage into the follicular lumen and the formation of vascular fibrin networks are characteristics of hemorrhagic anovulatory follicles [15]. Luteinized unruptured follicles are characterized by the visualization of a hazy, indistinct border between the follicle wall and antrum, thickening of the follicle wall and echotexture of the follicle wall which resembles that displayed by luteal tissue [15]. It is not uncommon for anovulatory follicles to develop to diameters which exceed normal pre-ovulatory diameters (i.e., > 25 mm) [15]. Anovulatory follicles typically regress over the course of a few weeks, but occasionally are sustained [15].

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Chapter 2

GENERAL OBJECTIVES AND HYPOTHESES

The general objectives of the studies contained in this thesis were to:

1) Characterize the development and regression of ovarian follicles and changes in circlulating concentrations of reproductively-active hormones during the menstrual cycle;
2) Characterize the development, ovulation and regression of ovarian follicles, and associated changes in endocrine levels, under the suppressive effects of oral contraception; and
3) Elucidate the effects of initiating oral contraceptive use at defined stages of follicle development.

The corresponding research hypotheses were tested:

1) Women exhibit major and minor waves of ovarian follicular development during the menstrual cycle, in association with changes in the concentrations of reproductively-active steroid and gonadotropin hormones;
2) Ovarian follicular development and ovulation during the use of oral contraception is a common phenomenon related to lack of endocrine suppression during the HFI, most notably in women taking low EE dose formulations ($\leq$ 20 μg EE); and
3) Dominant follicles secrete estradiol and become increasingly responsive to LH as they exert functional dominance over subordinate follicles after being physiologically selected for preferential growth.

Chapter 3

A NEW MODEL FOR OVARIAN FOLLICULAR DEVELOPMENT DURING THE HUMAN MENSTRUAL CYCLE

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3.1 Abstract

Objective: To evaluate changes in ovarian follicle dynamics during the human menstrual cycle to test the hypothesis that folliculogenesis occurs in a wave-like fashion.

Materials and Methods: Sixty-three healthy women of reproductive age (range=19-43) with a history of regular menstrual cycles, not taking medication(s) known to interfere with reproductive function, were enrolled in the study. Transvaginal ultrasonography was performed daily for one interovulatory interval (IOI) to monitor ovarian follicular development. Changes in the diameter and number of follicles $\geq$ 4 mm were evaluated each day during the IOI.

Results: Sity-eight percent of women exhibited 2 waves of follicle development during the IOI and 32% exhibited 3 waves. Waves were characterized by an increase and subsequent decrease in the number of follicles $\geq$ 5 mm occurring in association with the growth of $\geq$ 2 follicles to $\geq$ 6 mm. A day effect and day by wave interaction were detected in the mean diameter of the largest 3 follicles and the number of follicles $\geq$ 5 mm.

Conclusions: The follicular wave phenomenon in women provides a new model for ovarian function during the menstrual cycle, and will improve our understanding of the ovarian response to fertility and hormonal contraceptive regimes.

3.2 Introduction

The term 'wave' has been used ambiguously in the context of mammalian folliculogenesis and has led to confusion regarding our understanding of human ovarian function. Human follicular growth, in its entirety, begins at diameter of approximately 0.03 mm (i.e., primordial follicles) and continues for approximately 12 menstrual cycles until ovulation occurs [1]. Histologic evaluation of human ovarian follicular development has been interpreted to mean that the recruitment of 3 to 11 small antral follicles (2-5 mm) occurs in the late luteal phase of the menstrual cycle [2]. A single follicle is then selected from this cohort in the early follicular phase to undergo continued growth and ovulation at mid-cycle [3, 4]. Limited follicular development has

been thought to occur in the luteal phase due to the inhibitory effects of luteal progesterone [5-8].

Gougeon described 'waves' of ovarian follicular growth in women as the continuous entry of pre-antral resting follicles into the growing phase throughout the menstrual cycle [1]. Other authors have also referred to 'waves' of antral follicle development during the human menstrual cycle; however, no definitions of 'wave' are given [9-11]. Waves of folliculogenesis in animal models (eg., bovine, equine, ovine) are defined as the synchronous growth of a group of antral follicles [13]. The bovine estrous cycle, in particular, has been used as a model for the study of ovarian function in women [14]. Follicular waves emerge in cows at regular intervals throughout the estrous cycle, and are preceded by a rise in FSH [15]. Two and three waves of follicular development are most commonly observed during the bovine estrous cycle [14, 16, 17]. The final wave of follicular development in the estrous cycle of cows is ovulatory, while all preceding waves are anovulatory [14, 18]. It has been speculated that the wave phenomenon of follicular development in animal species permits a species-specific number of follicles to continue to grow and have the potential to ovulate while minimizing attrition from the follicular reserve by suppressing recruitment between waves [13].

In contrast to a wave pattern of development, it has been suggested that a single follicle grows by chance during a hormonally privileged period of the menstrual cycle in women [2]. According to this theory, referred to as the "Propitious Moment Theory" [19], antral follicles grow and regress continuously until conditions are right for a gonadotropin surge. The gonadotropin surge then induces ovulation of the follicle that, by happenstance, was mature at exactly the right point in the cycle.

High-resolution transvaginal ultrasonography is a very effective method of monitoring ovarian follicular growth in women [12]. Although this imaging tool does not allow observation of pre-antral and early antral (i.e., < 2 mm) follicles, it provides precise visualization of follicles at advanced stages of antral development during the last 2 weeks of follicle growth (i.e., ≥ 2 mm). Clinical observations in women undergoing transvaginal ultrasonographic ovarian monitoring in our laboratory revealed substantial follicular development during the luteal phase of the menstrual cycle. These

observations led to the notion that waves of ovarian follicular growth may occur in women, as documented in animal species. The objective of this study was to characterize the daily growth and regression of ovarian follicles in women during one interovulatory interval (IOI) to test the hypothesis that wave-like changes in the number and diameter of follicles during an IOI would be detected ultrasonographically.

3.3 Materials and Methods

Sixty-three women were enrolled in the study. Participants were assessed, by history and physical examination, to be healthy women of reproductive age (mean age $\pm$ SD = 28.0 $\pm$ 6.9 years, range = 19 - 43 years). Women who were pregnant or lactating within 6 months of enrolling in the study, had used hormonal contraception within 3 months of enrolling in the study, had a history of irregular menstrual cycles, were taking medication(s) known or suspected to interfere with reproductive function, or were planning surgery during the study period were not eligible to participate. Information about ethnicity, smoking status, body mass index, gravidity, parity and previous oral contraceptive use were obtained from each woman who took part in the study.

Each participant underwent daily transvaginal ultrasonographic evaluation of her ovaries for one IOI. An IOI was defined as the interval from one ovulation to the subsequent ovulation. Scans were initiated 12 days after menses (i.e., before the first ovulation) and were continued until 3 days after the second ovulation. Ovulation was defined as the disappearance of a large follicle ($\geq$15 mm) that had been identified by ultrasonography on the previous day and the subsequent visualization of a corpus luteum [20].

During each examination, follicles $\geq$ 2 mm were counted and measured. Follicles were first imaged in an approximately transverse plane. The image was frozen when the follicle appeared maximal, and the longest and widest follicle dimensions were recorded. The transducer was then rotated 90 degrees and similar measurements were recorded. Follicle diameter was estimated as the average of the 4 measurements. High-resolution ATL Ultramark 9 HDI and HDI 5000 ultrasound machines with 5-9 MHz multi-frequency convex array transducers (Advanced Technologies Laboratories; Bothell, WA, USA) were used to acquire follicular data. Scans were performed by a

single ultrasonographer (ARB) approximately ninety percent of the time; a second ultrasonographer (RAP) was available when the primary sonographer was not present. The study protocol was approved by the Institutional Review Board of the University of Saskatchewan and Saskatoon District Health.

Two methods were used to characterize changes in follicle diameter during the IOI: 1. the Identity Method and 2. the Non-Identity Method. The Identity Method [17, 21] involved drawing sketches of all follicles $\geq$ 4 mm in each ovary immediately following each scan. The day-to-day identities of individual follicles were determined using the internal iliac blood vessels, the ovarian hilus, and the location of neighboring follicles and the corpus luteum within the ovary as landmarks. The diameter profiles of individual follicles that grew to $\geq$ 8 mm throughout the IOI were graphed for each woman. Only follicles $\geq$ 8 mm were identified because the extraordinary number of 4 to 7 mm follicles made it difficult to accurately determine the day to day identities of these small follicles. The Non-Identity Method [22] involved sorting all follicles $\geq$ 4 mm in descending order of diameter for each woman on every day during the IOI. The diameters of the follicles occupying the largest, second largest, third largest categories etcetera were then plotted daily during the IOI, regardless of individual identity.

The number of follicles $\geq$ 5 mm detected on each day of the IOI were graphed for each woman. Follicle number data were combined for both ovaries, based on the results of studies in animal models [23-26] and analyses of follicle number data which compared right- versus left-side ovulations and whether dominant follicles in the luteal phase grew ipsilateral or contralateral to the CL. Peak to peak and trough to trough intervals in the number of follicles $\geq$ 5 mm were evaluated for each woman during the IOI to determine whether follicles grew in a wave-like fashion. A trough was defined as a data point immediately preceeded by at least 2 decreasing data points and immediately succeeded by at least 2 increasing data points. A peak was defined as the highest point between 2 troughs, preceded by an increasing trend and succeeded by a decreasing trend. When the peak or trough fell on either extreme of the x-axis, peaks succeeded by a decreasing trend and troughs preceded an increasing trend were considered. If a peak or trough occurred during a plateau, the first data point of the plateau was used.

An increase in the number of follicles $\geq$ 5 mm was defined as 2 successively increasing data points or 1 increasing data point followed by a plateau and an increasing trend thereafter. A decrease in the number of follicles $\geq$ 5 mm was defined as 2 successively decreasing data points or 1 decreasing data point followed by a plateau and a decreasing trend thereafter. When the increase or decrease originated or terminated in a plateau, the first data point in the plateau was used to demarcate the increase or decrease. An increase and subsequent decrease in the number of follicles $\geq$ 5 mm, occurring in association with the growth of at least 2 follicles to $\geq$ 6 mm, was considered a 'wave' of follicular development.

For statistical and illustrative purposes, follicle diameter and number data were normalized and centralized according to the number of follicle waves observed. Profiles of the mean diameter of the largest 3 follicles throughout the IOI, as determined by the Non-Identity method, were normalized to the mean IOI for women exhibiting 2 or 3 waves of follicle development during the cycle. Profiles of the number of follicles $\geq$ 5 mm for each woman were normalized in the same manner. Wave emergence was defined as the day on which the largest follicle of the wave was detected at 4-5 mm. Diameter profiles of the largest follicle of each wave were centralized to the mean day of emergence of each wave. The number of follicles $\geq$ 5 mm were centralized to the mean day of emergence of the first wave and 7 days before the emergence of the second wave for women with 2 waves. Follicle number data were centralized to the mean day of emergence of the first wave, 6 days before the emergence of the second wave and 3 days before the emergence of the third wave in women with 3 waves. Normalized and centralized follicle diameter and follicle number data were then truncated to day 27 (i.e., day 0 = 1^{st} ovulation), and repeated measures analyses of variance were used to determine an effect of day, wave, or a day by wave interaction (PROC MIXED, SAS/STAT Software, 2001).

Interovulatory intervals, interwave intervals, days of wave emergence and maximum follicle diameter were compared within and between women with 2 and 3 waves of follicle growth using independent sample T-tests, paired T-tests and Analyses of Variance with Scheffec's post hoc tests. Fisher's Exact Test was used to determine if

age, Body Mass Index, ethnicity, smoking, previous oral contraceptive use, gravidity, and parity influenced the number of waves exhibited during the IOI.

3.4 Results

Data from 13 of the 63 women enrolled in the study women were excluded from analyses because of ovarian irregularities: 1 woman had an IOI greater than 2 standard deviations from the mean, 4 women exhibited luteal phases shorter than 2 standard deviations from the mean, 1 woman had an ovarian dermoid cyst, and 7 women developed an anovulatory follicular cyst, hemorrhagic anovulatory follicle, or luteinized unruptured follicle during the study. The remaining 50 data sets were used to characterize follicular dynamics in the present study.

Individual follicular diameter profiles were compared using the Identity and Non-Identity Methods (Figure 3.1 (A,B), Figure 3.2 (A,B)). The Non-Identity Method was chosen to characterize diameter profiles because it provided information about follicles in the 4-7 mm range, which the Identity Method did not.

No differences were detected between the proportions of right- versus left-side ovulations in women with 2 and 3 follicular waves ($p>0.05$). Likewise, we did not detect any differences between the numbers or diameters of dominant follicles which grew ipsilateral or contralateral to the CL during the luteal phase ($p>0.05$). Therefore, follicle number data were combined between ovaries.

Non-random changes in the number of follicles $\geq$ 5 mm and the diameter of follicles $\geq$ 6 mm (Non-Identity Method) were observed in all 50 women during the IOI, indicating a wave pattern of follicle development. Thirty-four of the 50 women (68%) exhibited 2 waves of follicle development; the remaining 16 women (32%) exhibited 3 waves of follicle development. The final wave of the cycle was ovulatory and the preceding waves were anovulatory in all 50 women. None of the women evaluated exhibited only a single wave of follicle development during the IOI.

A day effect ($p<0.0001$) and a day by wave interaction ($p<0.0001$) were detected in the mean diameter of the largest 3 follicles throughout the IOI as determined by the

Non-Identity Method, indicating that the profiles for the mean diameter of the largest 3 follicles were different in women with 2 waves as compared to women with 3 waves.

A day effect ($p<0.0001$) and a wave effect ($p=0.02$) were detected in the number of follicles $\geq$ 5 mm throughout the IOI. When follicle number data were centralized to the day of wave emergence, a significant day by wave interaction was detected ($p=0.01$), indicating that the changes observed in the number of follicles $\geq$ 5 mm in women with 2 waves differed from the changes observed in women with 3 waves during the IOI. Superimposed follicle diameter and number data in women with 2 versus 3 waves are illustrated in Figure 3.3 (A,B).

In evaluating the mean follicle number and diameter profiles in Figure 3.3 (A), 2 waves were visualized during the IOI. In Figure 3.3 (B), 3 waves in follicle diameter data were observed, while only 2 waves in follicle number data were apparent during the IOI. Individual follicle number profiles for women with 3 waves did exhibit 3 troughs followed by 3 peaks. However, the mean decrease in follicle number during wave 2 and the mean increase in follicle number during wave 3 overlapped making it appear that only 2 waves in follicle number were observed.

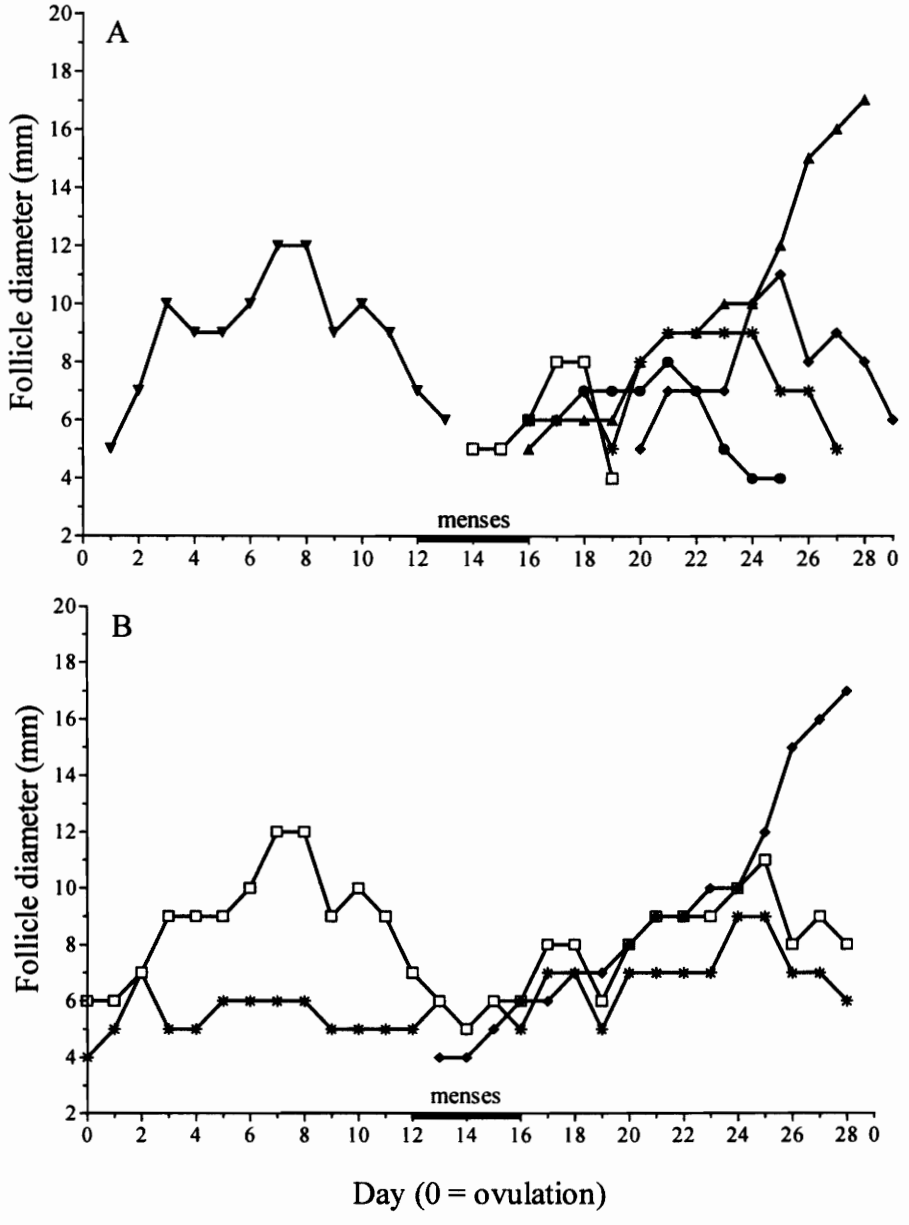

Figure 3.1: Follicular diameter profiles for a woman who exhibited 2 follicular waves during the IOI as determined by the (A) Identity Method and (B) Non-Identity Method. Note that only follicles that grew to $\geq$ 8 mm could be individually identified from day-to-day using the Identity Method. Follicles that grew to $\geq$ 6 mm were identified using the Non-Identity Method. In Figure 3.1(A) each follicle is represented by a different symbol. In figure 3.1(B) ◆ = largest follicle, □ = second largest follicle, and * = third largest follicle.

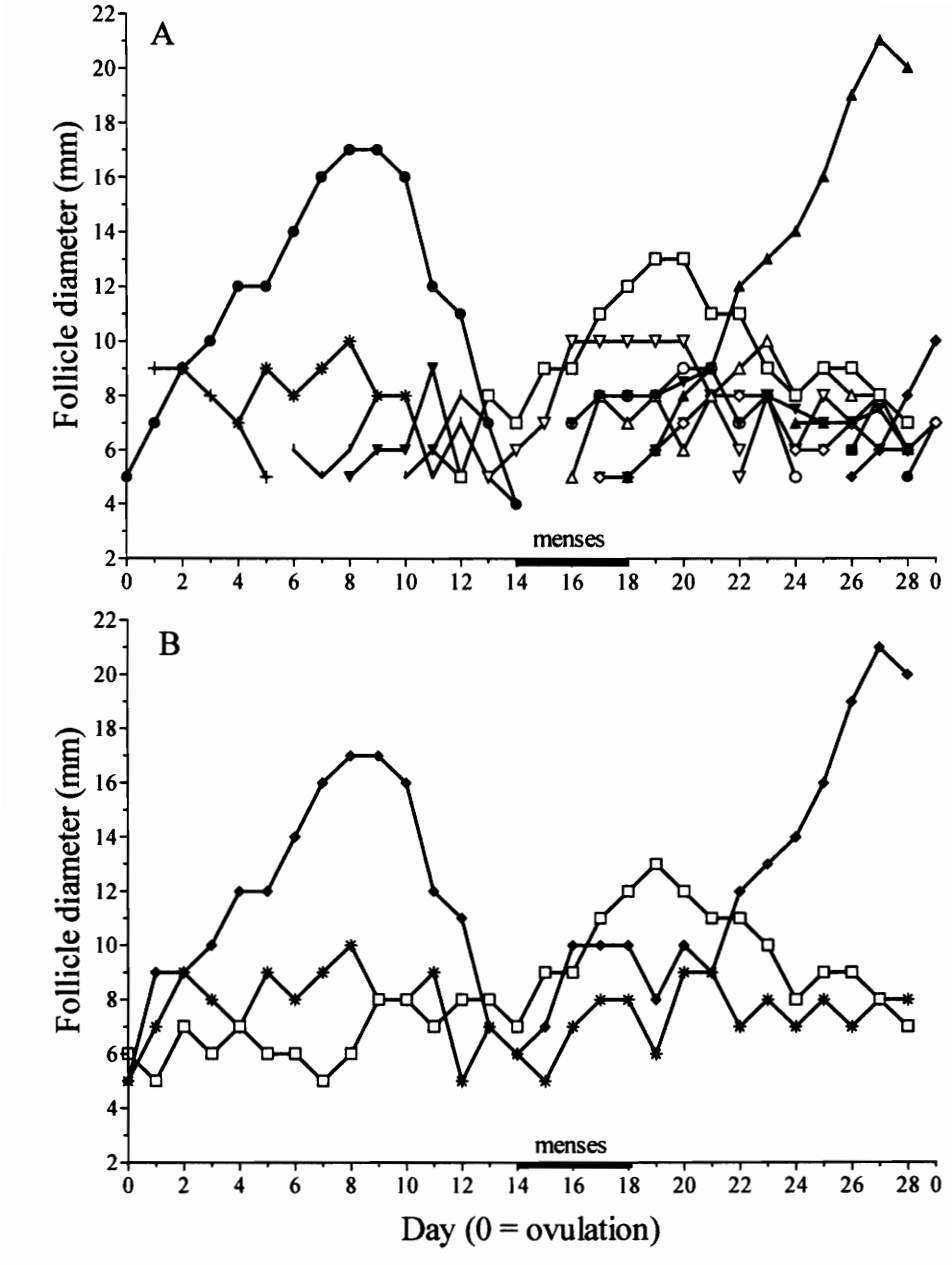

Figure 3.2: Follicular diameter profiles for a woman who exhibited 3 follicular waves during the IOI as determined by the (A) Identity Method and (B) Non-Identity Method. Note that only follicles that grew to $\geq$ 8 mm could be individually identified from day-to-day using the Identity Method. Follicles that grew to $\geq$ 6 mm were identified using the Non-Identity Method. In Figure 3.2 (A) each follicle is represented by a different symbol. In figure 3.2 (B) ◆ = largest follicle, □ = second largest follicle, and * = third largest follicle.

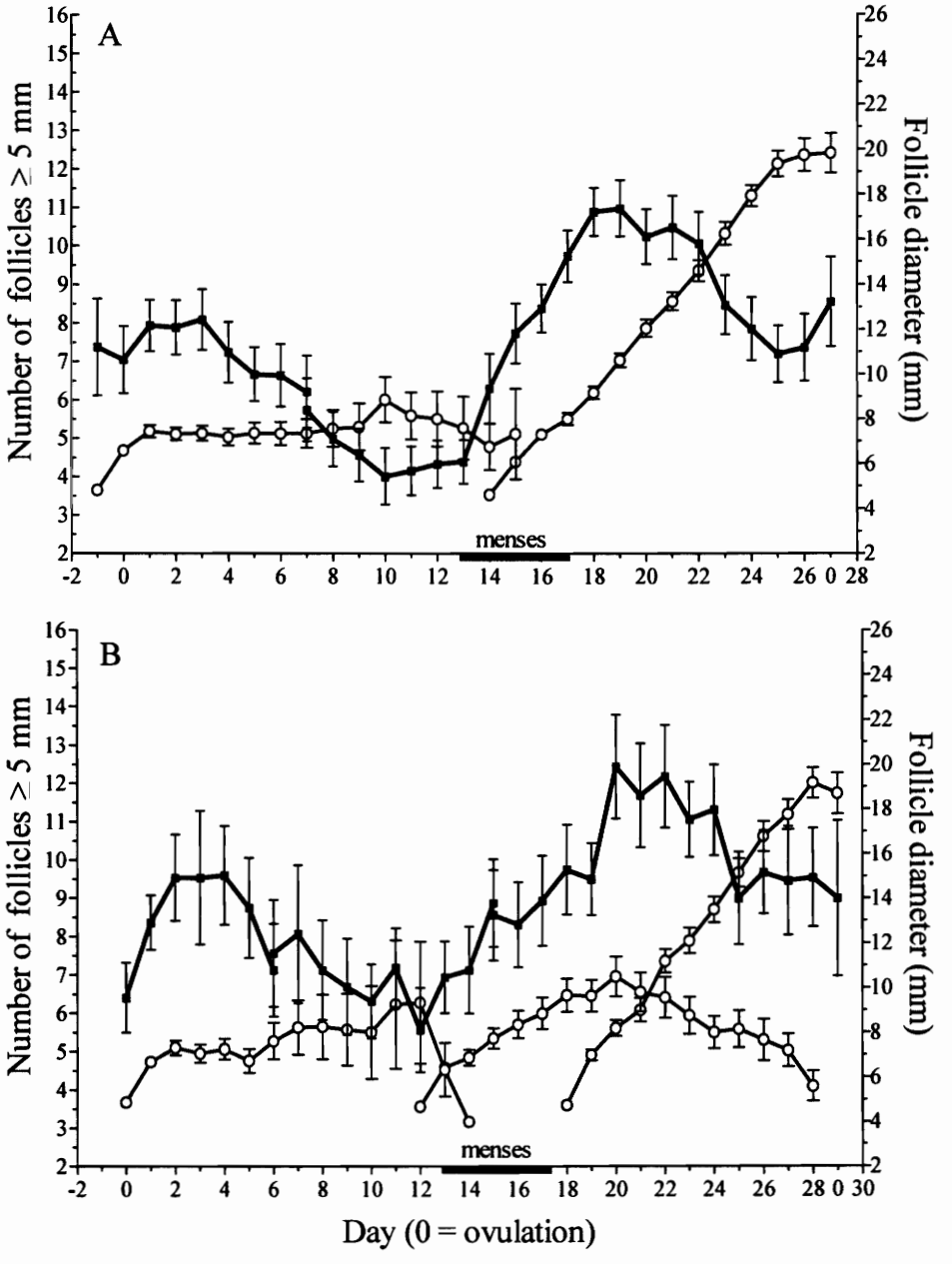

Figure 3.3: Day to day profiles (mean ± SEM) of the number of follicles ≥ 5 mm detected (■) and the diameter of the largest follicle of each wave (Non-Identity Method, o) for women exhibiting 2 waves (A; n=34) and 3 waves (B; n=16) during one IOI. Asterisks indicate follicle number data overlap.

The mean intervals between peaks (10.0 ± 0.4 days) and troughs (9.7 ± 0.6 days) were not different in women with 3 waves (p=0.70). However, the peak to peak interval of 16.0 ± 0.7 days in women with 2 waves was longer than the trough to trough interval of 13.7 ± 0.5 days (p=0.008).

In women with 2 wave cycles, the IOI was shorter ($p<0.05$) and the interwave interval was longer ($p<0.05$) than in women with 3 wave cycles (Table 3.1). The day of emergence of the first follicular wave was similar between women with 2 and 3 waves, but the second wave emerged earlier ($p<0.05$) in women with 3 waves as compared to women with 2 waves (Table 3.1).

Table 3.1: Characteristics (mean ± SEM) of ovarian follicular waves during the menstrual cycle in women.

	2 Waves (n=34)		3 Waves (n=16)		
	Wave 1	*Wave 2*	*Wave 1*	*Wave 2*	*Wave 3*
Interovulatory Interval (days)	27.4±0.4[a] (24-32)			29.4±0.6[b] (26-34)	
Interwave Interval (days)	14.7±0.4[a] (10-20)	------	11.9±0.8[b] (6-19)	6.6±0.4[c] (5-11)	------
Day of Wave Emergence	-0.5±0.3[a] (-3 - +3)	14.2±0.3[c] (+11 - +18)	-0.3±0.5[a] (-5 - +3)	11.6±0.6[b] (+6 - +15)	18.2±0.6[d] (+14 - +23)
Maximum Follicle Diameter (mm)	9.0±0.4[a] (5-16)	21.7±0.4[b] (17-26)	9.2±0.8[a] (6-17)	9.7±0.7[a] (6-15)	20.7±0.5[c] (17-25)

[a,b,c,d]: Within rows, values with no common superscript are different as determined by t-tests, paired t-tests and Analysis of Variance with Scheffe's post hoc tests ($p<0.05$)

Ovulatory follicles emerged in the early follicular phase and grew to a smaller pre-ovulatory diameter in 3 wave cycles than in 2 wave cycles ($p<0.05$, Table 3.1). Anovulatory follicular waves emerged one day before ovulation in women with 2 waves and on the day of ovulation and in the late luteal phase in women with 3 waves (Table 3.1). No differences were detected in the maximum diameter of the largest follicle of anovulatory waves in 2 wave and 3 wave cycles ($p>0.05$, Table 3.1). The mean diameters of follicles from anovulatory waves were smaller than follicles from ovulatory waves in both 2 and 3 wave cycles ($p<0.05$, Table 3.1). However, it is noteworthy that 4/66 (6%) anovulatory follicles grew to a pre-ovulatory diameter $\geq$ 15 mm and 20/66 (30%) grew to $\geq$ 10 mm.

Age, Body Mass Index, ethnicity, smoking, previous oral contraceptive use, gravidity, or parity were not found to influence the number of waves detected during the IOI ($p>0.05$).

3.5 Discussion

Our results supported the hypothesis that follicular development in women occurs in a wave-like fashion during the menstrual cycle. We observed non-random wave-like changes in follicle number and diameter and confirmed that women exhibit 2 or 3 waves of folliculogenesis during an IOI. This knowledge challenges the previously held notion that a single cohort of antral follicles grows only during the follicular phase of the menstrual cycle. The Identity and Non-Identity Methods of evaluating follicular dynamics in women during the IOI gave comparable results, similar to findings in animal models [22]. The Non-Identity Method, however, was more useful for tracking the development of follicles < 8 mm, because it did not require maintaining the day to day identities of the multitude of small (4-7 mm) follicles.

Peak to peak and trough to trough intervals in follicle number for women with 3 follicular waves were not different, which supported a wave theory of follicle development. We did not observe similar peak to peak and trough to trough intervals in women with 2 follicular waves compared to women with 3 waves. We attributed this inconsistency in 2 wave cycles to error in determining the day of emergence of the first wave. The first wave emerged, on average, one day before ovulation in 2 wave cycles.

Peaks in follicle number were often detected on the day of ovulation. Serial data were not available for all women on the days prior to the first ovulation. Therefore the first peak in follicle number may have occurred earlier or later than we could detect accurately.

A greater number of follicular waves during the cycle was associated with a longer interovulatory interval and shorter interwave interval. Only the final wave of follicle development was ovulatory, while all preceding waves were anovulatory. In both 2 and 3 wave cycles, the ovulatory wave emerged in the early follicular phase and anovulatory waves developed in the luteal phase. These observations were consistent with follicle wave dynamics as previously documented in animal models, particularly the bovine and equine models [13, 14, 27].

We postulated that the development of anovulatory follicles in the luteal phase occurred as a result of progesterone-mediated inhibition of LH secretion to levels that allowed follicular development to proceed to the antral or late antral stage, but did not allow the LH surge and ovulation to occur. Anovulatory follicles did not grow as large, on average, as ovulatory follicles. However, a notable number of women exhibited anovulatory follicles which grew to an ostensibly pre-ovulatory diameter. It could therefore be speculated that follicles developing in the luteal phase of the cycle have the potential to ovulate in the presence of an LH surge.

Previous studies in women have documented a greater incidence of right-side ovulations [28] while others have reported no difference [29]. However, we did not detect a difference in the number of right- versus left-side ovulations. It has also been reported that the CL exerted a negative effect on follicular growth in women [30]. The results of our study, and several other studies in animal models [23-26], however, do not support a negative effect of the CL on follicular growth. We did not detect differences in the numbers or diameters of dominant follicles which grew ipsilateral or contralateral to the CL during the luteal phase. We interpret these results to mean that the 2 ovaries act primarily as a single unit, and that ovarian follicular waves are regulated by systemic rather than local mechanisms as documented in the bovine model [26].

We did not detect effects of age, Body Mass Index, ethnicity, smoking, previous oral contraceptive use, gravidity, and parity on the number of waves exhibited during the IOI. However, it is noteworthy that our failure to detect differences in the number of follicular waves in women of different ages and body mass indices may have been due to small numbers of women ≤ 20 and > 35 years of age and women with body mass indices < 20 and > 35. Additional studies must be performed on women fitting broader demographic profiles before conclusions may be drawn.

Further evaluation of these data are being performed in our laboratory to determine the role of the pituitary gonadotropins and ovarian steroid hormones in the development of ovarian follicular waves in women. These studies will help us to understand the mechanisms regulating the development of follicular waves during the menstrual cycle, as well as follicular development and ovulations observed during hormonal contraceptive use [31-36].

Documentation of a wave phenomenon of ovarian follicular development in women provides a new model for folliculogenesis during the human menstrual cycle. We anticipate that the knowledge of follicular waves during the menstrual cycle will have profound implications for infertility diagnoses and treatment in women. The development of more than one wave of follicular development during a woman's cycle may provide women undergoing assisted reproduction with more opportunities for initiating ovarian stimulation protocols. This option would provide women with a more time-efficient and less expensive treatment regimen. Consideration of a wave model for ovarian follicular growth may also be useful for the development of more efficacious and user-friendly hormonal contraceptive regimens.

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Chapter 4

CHARACTERIZATION OF OVARIAN FOLLICULAR WAVE DYNAMICS IN WOMEN

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4.1 Abstract

Objective: A wave phenomenon of ovarian follicular development in women has recently been documented in our laboratory. The objective of the present study was to characterize ovarian follicular waves to determine whether women exhibit major and minor wave patterns of follicle development during the interovulatory interval (IOI).

Materials and Methods: The ovaries of 50 women with clinically normal menstrual cycles were examined daily using transvaginal ultrasonography for one IOI. Profiles of the diameters of all follicles $\geq$ 4 mm and the numbers of follicles $\geq$ 5 mm were graphed during the IOI. Major waves were defined as those in which one follicle grew to $\geq$ 10 mm and exceeded all other follicles by $\geq$ 2 mm. Minor waves were defined as those in which follicles did not develop to 10 mm and follicle dominance was not manifest. Blood samples were drawn to measure serum concentrations of estradiol-17β, LH and FSH.

Results: Women exhibited major and minor patterns of follicular wave dynamics during the IOI. Of the 50 women evaluated, 29/34 women with 2 follicle waves (85.3%) exhibited a minor-major wave pattern of follicle development and 5 women (14.7%) exhibited a major-major wave pattern. Ten of the 16 women with 3 follicle waves (62.5%) exhibited a minor-minor-major wave pattern, 3 women (18.8%) exhibited a minor-major-major wave pattern and 3 women (18.8%) exhibited a major-major-major wave pattern.

Discussion: Documentation of major and minor ovarian follicular waves in women challenges the traditional theory that a single cohort of antral follicles is recruited for growth only during the follicular phase of the menstrual cycle.

4.2 Introduction

Ovarian follicular development in women during the menstrual cycle has not been fully elucidated. The traditional theory of human folliculogenesis, developed over the past 50 years, holds that a single cohort of 3-11 antral follicles is recruited to grow in each ovary during the late luteal phase of the human menstrual cycle [1]. A single dominant follicle is believed to be selected from this cohort for preferential growth in the early to mid follicular phase. The dominant follicle continues to develop and

ovulate, while all other subordinate follicles regress [2-4]. Follicular development to an ostensibly ovulatory diameter in women has been believed to occur exclusively in the follicular phase [5-7], while follicular quiescence has been thought to occur during the luteal phase due to the inhibitory effects of luteal progesterone production [8-13]. Antral follicles have been reported in the luteal phase of the menstrual cycle occasionally [14-16]. However, only 6% of follicles in luteal phase were believed to be healthy, as determined by oocyte viability and granulosa cell number [10]. In other reports, it appeared as though luteal phase follicular development represented an abnormal reproductive event [14-16].

Much of the current understanding of ovarian follicular development during the menstrual cycle has been based on earlier studies in which measurements of urine and serum endocrine levels were used to indirectly assess follicle growth and ovulation [17]. Histologic evaluation of ovaries during laparotomy and laparoscopy or following ovariectomy have also been reported, sometimes in association with endocrine measurements [9, 10, 14, 18-29]. Transabdominal and transvaginal ultrasonography have also been used intermittently during the menstrual cycle (usually only during the follicular phase) to assess follicle development [15, 16, 30-54]. The traditional model of human folliculogenesis have been proposed based on limited studies in women and extrapolations from studies performed in rodents, non-human primates and domestic animal species [7, 26, 55-63].

Successive waves of ovarian follicular development during the menstrual cycle have recently been documented in our laboratory using serial high-resolution transvaginal ultrasonographic evaluation of follicle growth and regression [64]. Thirty-four of fifty women (68%) with normal menstrual cycles exhibited 2 waves of follicular development during an interovulatory interval and 16 women (32%) exhibited 3 waves [64]. Only the final wave of each cycle was ovulatory; all preceding waves were anovulatory. Three-wave cycles were associated with longer interovulatory intervals and shorter interwave intervals than 2-wave cycles [64]. Documentation of a follicular wave phenomenon in women has challenged the traditional notion that a single cohort of antral follicles grows only during the follicular phase of the menstrual cycle, and

provided a new model for studying ovarian follicular development during the menstrual cycle.

The development of 2 and 3 ovarian follicular waves in women [64] is comparable to the follicular wave phenomenon described in domestic animal species [65, 66]. In the cow, 2 and 3 waves of follicular development were observed during the estrous cycle [67-71]. The final wave was ovulatory, while all preceding waves were anovulatory. In the mare, major and minor follicular waves have been documented [72]. Major follicular waves were defined as the synchronous growth of a group of follicles followed by selection of a follicle for continued growth, and regression of all other 'subordinate' follicles of the wave. Major waves were either anovulatory or ovulatory, depending on the stage of the cycle during which they developed. Minor follicle waves in mares were characterized by the failure of any one follicle to become dominant over other follicles of the wave.

Follicle wave dynamics in domestic animals have been associated with cyclic changes in reproductive steroid and glycoprotein hormones. The emergence of follicular waves in cows [73] and mares [72] was preceded by a rise in the circulating concentrations of FSH. After wave emergence, selection of a dominant follicle was associated with a decline in circulating concentrations of FSH, acquisition of granulosa LH receptors and rising circulating concentrations of estradiol [74-79]. Progesterone production from the corpus luteum in cows elicited a suppressive effect on LH and inhibited ovulation of dominant follicles from waves that developed in the luteal phase [80, 81].

The objective of the present study was to characterize ovarian follicular wave activity in women, by evaluating changes in follicle diameter, number of follicles > 5 mm, and circulating concentrations of FSH, LH and estradiol during one interovulatory interval. We hypothesized that women would exhibit major and minor patterns of follicle wave development.

4.3 Materials and Methods

Data obtained from 50 women enrolled in our initial study which documented 2 and 3 ovarian follicular waves [64] were carefully evaluated to elucidate different

patterns of follicle wave development. Participants were assessed, by history and physical examination, to be healthy women of reproductive age (mean ± SD = 28.0 ± 6.9 years, range = 19 - 43 years). Women who were currently or recently pregnant or lactating, had used hormonal contraception within 3 months of enrollment, had a history of irregular menstrual cycles, or were taking medication(s) known or suspected to interfere with reproductive function were not eligible to participate. Informed consent was obtained from all women prior to initiating study procedures. Study protocol was approved by the Institutional Review Board of the University of Saskatchewan.

Each volunteer underwent daily transvaginal ultrasonographic evaluation of her ovaries for one interovulatory interval (IOI). An IOI was defined as the interval from one ovulation to the subsequent ovulation. Ultrasound examinations were initiated 12 days after menses (i.e., a few days before the first ovulation) and were continued until 3 days after the second ovulation. Ovulation was defined as the disappearance of a large follicle (> 15 mm) that had been identified by ultrasonography on the previous day, and the subsequent visualization of a corpus luteum [41, 82]. Follicles ≥ 4 mm were measured during each examination, and the number of follicles ≥ 5 mm tabulated. The length and width of each follicle were measured in both the sagittal and transverse planes. Follicle diameter was then calculated by averaging the mean measurement in the sagittal plane by the mean measurement in the transverse plane. The methods used for tracking follicle diameter and follicle number each day during the IOI are as described in Baerwald et al. [64].

High-resolution Ultramark 9 and ATL HDI 5000 ultrasound machines with 5-9 MHz multi-frequency convex array transducers (Advanced Technologies Laboratories: Bothell, WA, USA) were used to acquire follicular data. Approximately 90% of the examinations were performed by one sonographer (ARB). A second sonographer (RAP) was available when the primary sonographer was not available.

Follicular waves were characterized by an increase and subsequent decrease in the number of follicles ≥ 5 mm, occurring in association with the growth of at least 2 follicles to ≥ 6 mm, as documented in the previous report [64]. In the present analysis, major waves were defined as waves in which one follicle grew to ≥ 10 mm and exceeded the next largest follicle by ≥ 2 mm (i.e., development of a dominant follicle).

Minor waves were defined as those in which the largest follicle developed to < 10 mm and did not grow larger than all other follicles of the wave by ≥ 2 mm (i.e., no evidence of follicular dominance). Wave emergence was defined as the day at which the largest follicle of each wave was first identified, retrospectively, at 4-5 mm. An interwave interval (IWI) was defined as the interval from the emergence of one wave to the emergence of the subsequent wave. Selection was defined as the day on which the prospective dominant follicle became, and remained, larger than all other follicles of a major wave.

Blood samples were drawn every third day during the IOI in a stratified manner among women so that each day of the IOI was represented. The stratification scheme was used to randomly assign one third of the women to have blood drawn on days 1, 4, 7 etcetera, one third on days 2, 5, 8 etcetera, and the remaining one third on days 3, 6, 9 etcetera. Blood was collected into a 7 mL clot-activated tube and allowed to sit at room temperature for 15-30 minutes before centrifugation for 10 minutes at 3000 rpm (700G). The serum was drawn off and stored at –20°C. Sequential competitive fluorescence immunoassays (Immulite®, Diagnostic Products Corporation, Los Angeles) were performed to measure serum concentrations of Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH) and estradiol-17β (E2). Inter-assay co-efficients of variation were as follows: LH (low=6.3%, medium=4.0%, high=4.5%), FSH (low=8.0%, medium=2.9%, high=4.1%) and E2 (low=9.8%, medium=5.6%, high=4.3%). Minimal detectable limits were 0.1 mIU/mL for FSH, 0.1mIU/mL for LH and 15 pg/mL for E2.

We initially categorized follicle diameter, follicle number and endocrine data into 2- or 3-wave patterns [64]. In the present study, data were further partitioned into major and minor wave patterns: minor major (- +), major major (+ +), minor minor major (- - +), minor major major (- + +) and major major major (+ + +). Follicle and endocrine data were centralized to the day of wave emergence and normalized to the mean IOIs of the respective wave patterns.

In women with 2 follicular waves, T-tests were used to compare - + and + + wave cycles with respect to days of wave emergence, IOI, IWI, maximum number of follicles > 5mm, follicle diameter, growth rate, and regression rate (SPSS Version 11,

2002). In women with 3 follicular waves, analyses of variance with Scheffe's post hoc tests were used to compare endpoints between - - +, - + + and + + + wave cycles (SPSS Version 11, 2002). T-tests and repeated measures analyses (SPSS Version 11, 2002; PROC MIXED, SAS/STAT Software, 2002) were used to compare IWI, follicle diameter, growth rate, and regression rate between waves. Repeated measures analyses (PROC MIXED, SAS/STAT Software, 2002) were also used to assess changes in follicle diameter, follicle number and endocrine status during the IOI.

4.4 Results

The proportions of women exhibiting major and minor wave patterns and the respective interovulatory and interwave intervals are shown in Table 4.1. Waves of follicle development emerged on days 0 (i.e., day of 1st ovulation) and 14 (- +), -1 and 14 (++), -1, 11 and 17 (- - +), -1, 13 and 20 (- + +) and 1, 13 and 19 (+++). The days of wave emergence were not different between women with - + and + + wave patterns ($p>0.05$), or among women with - - +, - + + and + + + wave patterns ($p > 0.05$).

Follicle diameter and number data during the IOI for the 2- and 3-wave patterns are shown in Figures 4.1 (A,B) and 4.2 (A,B,C) respectively (day effect: $p<0.000$). A nadir in follicle number was detected prior to the emergence of all major and minor waves.

Table 4.1: Interovulatory intervals (IOI) and interwave intervals (IWI) (mean ± SEM) for women with 2- and 3-wave patterns of follicular development during the ovarian cycle.

Pattern	Proportion	IOI (days)	IWI (days) Wave 1	Wave 2	Wave 3
2 Waves					
- +	29/34 (85%)	27.4±0.4^{m}	14.6±0.5 ^{a}	13.1±0.7^{b}	---
+ +	5/34 (15%)	27.2±1.0^{m}	15.2±0.7 ^{a}	13.2±0.4^{b}	---
Overall	34/50 (68%)	27.4±0.4	14.7±0.4	13.1±0.3	---
3 Waves					
- - +	10/16 (63%)	28.8±0.7^{o}	11.3±1.2^{c}	6.7±0.7^{d}	11.4±0.7^{e}
- + +	3/16 (19%)	30.7±1.8^{o}	13.7±0.9^{c}	6.7±0.3^{d}	11.0±1.2^{e}
+ + +	3/16 (19%)	30.0±1.0^{o}	12.3±0.3^{c}	6.0±0.6^{d}	10.7±0.3^{e}
Overall	16/50 (32%)	29.4±0.6	11.9±0.8	6.6±0.4	11.2±0.4

IWI: ^{a,b} Within rows for 2 wave patterns, values with no common superscript are different.
^{c,d,e} Within rows for 3 wave patterns, values with no common superscript are different.
IOI; ^{m,n} Within columns for 2 wave patterns, values with no common superscript are different.
^{o,p} Within columns for 3 wave patterns, values with no common superscript are different.
Significance indicated at $p<0.05$.

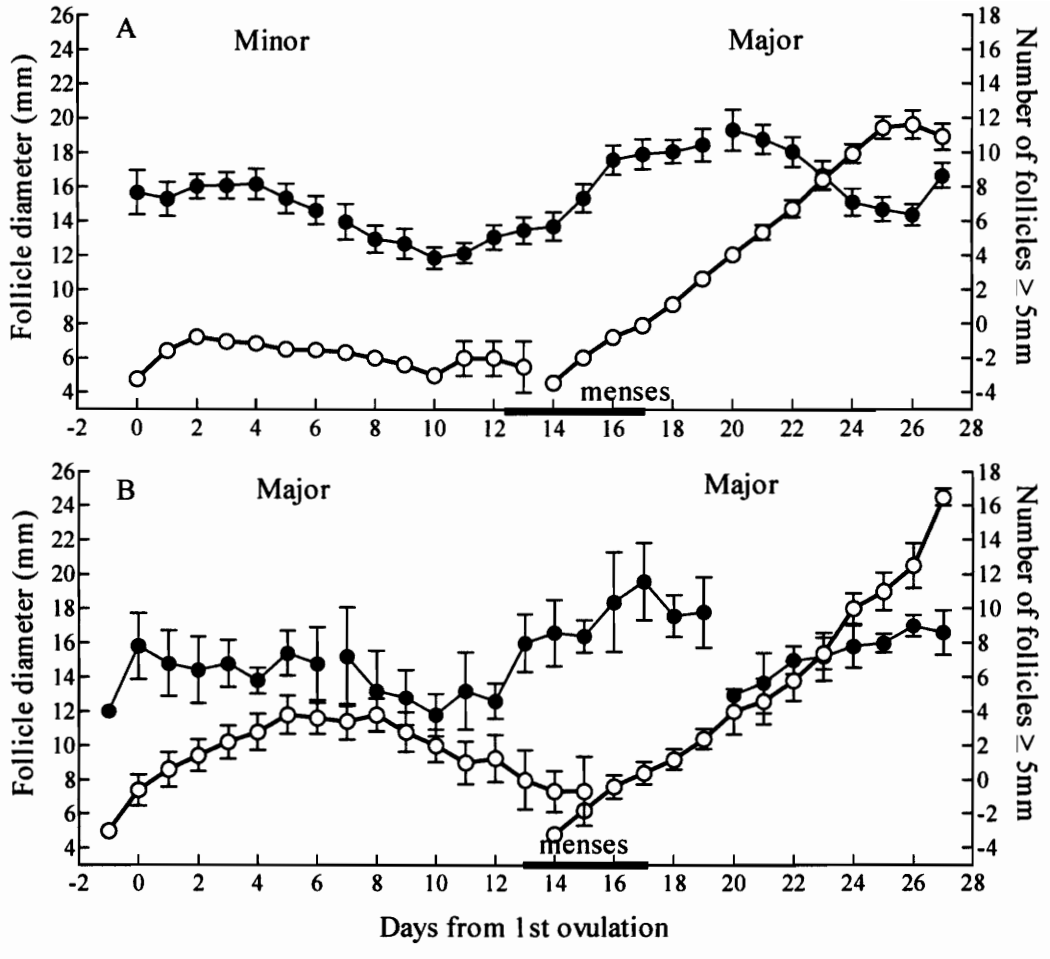

Figure 4.1: Diameter of the largest follicle of each wave (○) and the number of follicles ≥ 5mm (●) in women with 2-wave interovulatory interval (IOI). The majority of women exhibited a - + wave pattern (A; n=29); all other women exhibited a + + wave pattern (B; n=5). Follicle diameter and number data were normalized to the mean IOI for each wave pattern and centralized to wave emergence. Follicle number data were displayed from emergence of the first wave and midpoint of both waves.

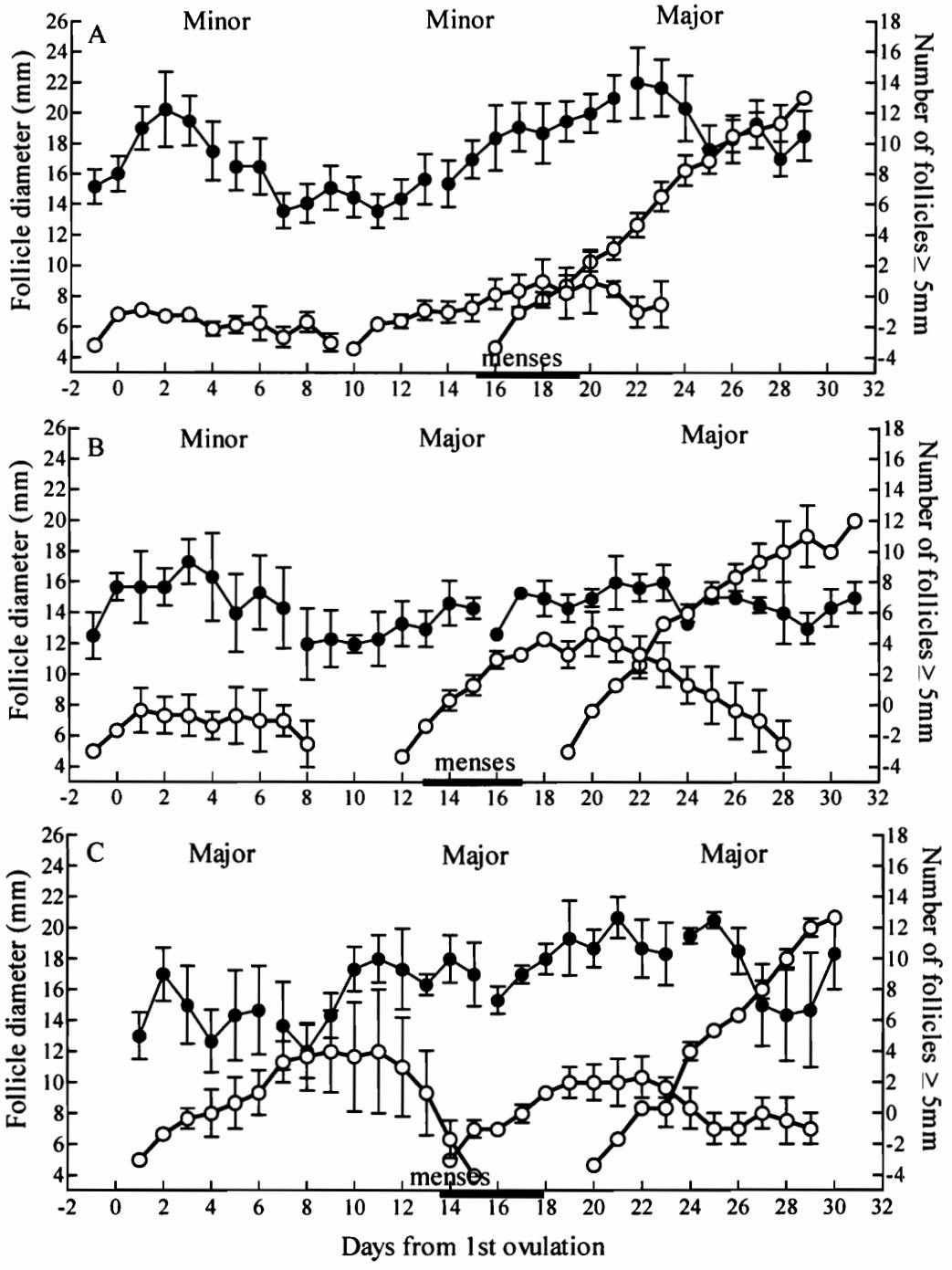

Figure 4.2: Diameter of the largest follicle of each wave (○) and the number of follicles ≥ 5mm (●) in women with 3-wave interovulatory interval (IOI). Women exhibited either a - - + wave pattern (A; n=10; diameter data centralized to day 0,), - + + wave pattern (B; n=3) or a + + + wave pattern (C; n=3). Follicle diameter and number data were normalized to the mean IOI for each wave pattern. Follicle number data were displayed from emergence of the first wave and midpoint of all 3 waves.

Peak diameters of the largest, second largest and third largest follicles of each wave for the 5 different wave patterns are shown in Table 4.2. The peak diameters of the largest follicles from major waves were greater than those in minor waves ($p<0.05$), and dominant follicles from major ovulatory waves were larger than those in major anovulatory waves ($p<0.05$) in all 5 patterns of follicular growth.

Table 4.2: Maximum diameter (mean ± SEM) of the largest (F1), 2nd largest (F2) and 3rd largest (F3) follicles of each wave in women with 2- and 3-wave patterns of follicular development during the ovarian cycle.

	1st wave			2nd wave			3rd wave		
Pattern	F1	F2	F3	F1	F2	F3	F1	F2	F3
2 Waves									
- +	7.8±0.2[a]	7.3±0.2[a]	6.9±0.2[a]	21.9±0.4[a]	9.8±0.3[a]	8.7±0.3[a]	–	–	–
+ +	12.8±0.9[b]	8.0±0.4[a]	6.8±0.7[a]	21.0±1.5[a]	10.6±0.7[a]	8.4±0.4[a]	–	–	–
Overall	8.6±0.4	7.4±0.2	6.9±0.2	21.7±0.4	9.9±0.3	8.7±0.2	–	–	–
3 Waves									
- - +	8.0±0.3[c]	7.3±0.3[c]	6.9±0.2[c]	8.2±0.6[c]	7.5±0.6[c]	6.8±0.5[c]	20.5±0.8[c]	9.0±0.8[c]	7.9±0.2[c]
- + +	8.7±1.2[c]	8.3±1.5[c]	7.3±1.2[d]	13.3±0.9[c]	8.7±1.7[c]	7.7±1.2[c]	20.0±0.6[c]	10.0±1.5[c]	7.3±0.3[d]
+ + +	14.3±2.2[d]	8.7±1.9[c]	7.7±1.3[d]	11.3±0.9[c]	9.0±0.6[c]	8.0±0.6[c]	21.0±0[c]	10.7±0.3[c]	9.0±0.6[e]
Overall	9.3±0.8	7.8±0.4	7.1±0.3	9.8±0.7	8.0±0.5	7.2±0.4	20.5±0.5	9.5±0.5	8.0±0.2

[a,b] Within columns for 2 wave patterns, values with no common superscripts are different.
[c,d,e] Within columns for 3 wave patterns, values with no common superscripts are different.
Significance indicated at $p<0.05$.

Follicle diameter and number for major and minor waves are compared in Figure 4.3 (A,B). In major waves, more follicles were recruited to grow and the largest follicle of the wave reached a greater peak diameter than in minor waves (follicle number; day effect: $p<0.0001$, wave effect: $p<0.0001$, day*wave effect: $p=0.64$), (follicle diameter; day effect: $p<0.0001$, wave effect: $p<0.0001$, day*wave effect: $p<0.0001$). The mean diameter of all follicles $\geq$ 6 mm and number of follicles $\geq$ 5 mm

increased simultaneously (r = +0.99) in major waves, until day 4 when follicle diameter continued to increase and number decreased to day 11 (r = -0.68). In contrast, follicle diameter and number data increased and decreased simultaneously throughout the duration of minor waves (r = +0.87). The growth rate of the largest follicle in major waves was slower than that in minor waves ($p<0.05$, Table 4.3). No difference in regression rates was detected ($p>0.05$, Table 4.3).

Table 4.3: Characteristics (mean ± SEM) of major and minor follicular waves during the ovarian cycle in women.

	Major Waves (n=67)	Minor Waves (n=52)
Maximum number follicles ≥ 5mm	13.9±0.7[a]	11.2±0.7[b]
Maximum follicle diameter (mm)	19.5±0.5[a]	8.0±0.2[b]
Growth rate of largest follicle from emergence at 4-5mm (mm/day) to maximum diameter	1.4±0.04[a]	1.8±0.1[b]
Regression rate of largest follicle to 4-5 mm (mm/day) from maximum diameter	-1.2±0.1[a]	-1.0±0.1[a]

[a,b] Within rows, values with no common superscripts are different ($p<0.05$).

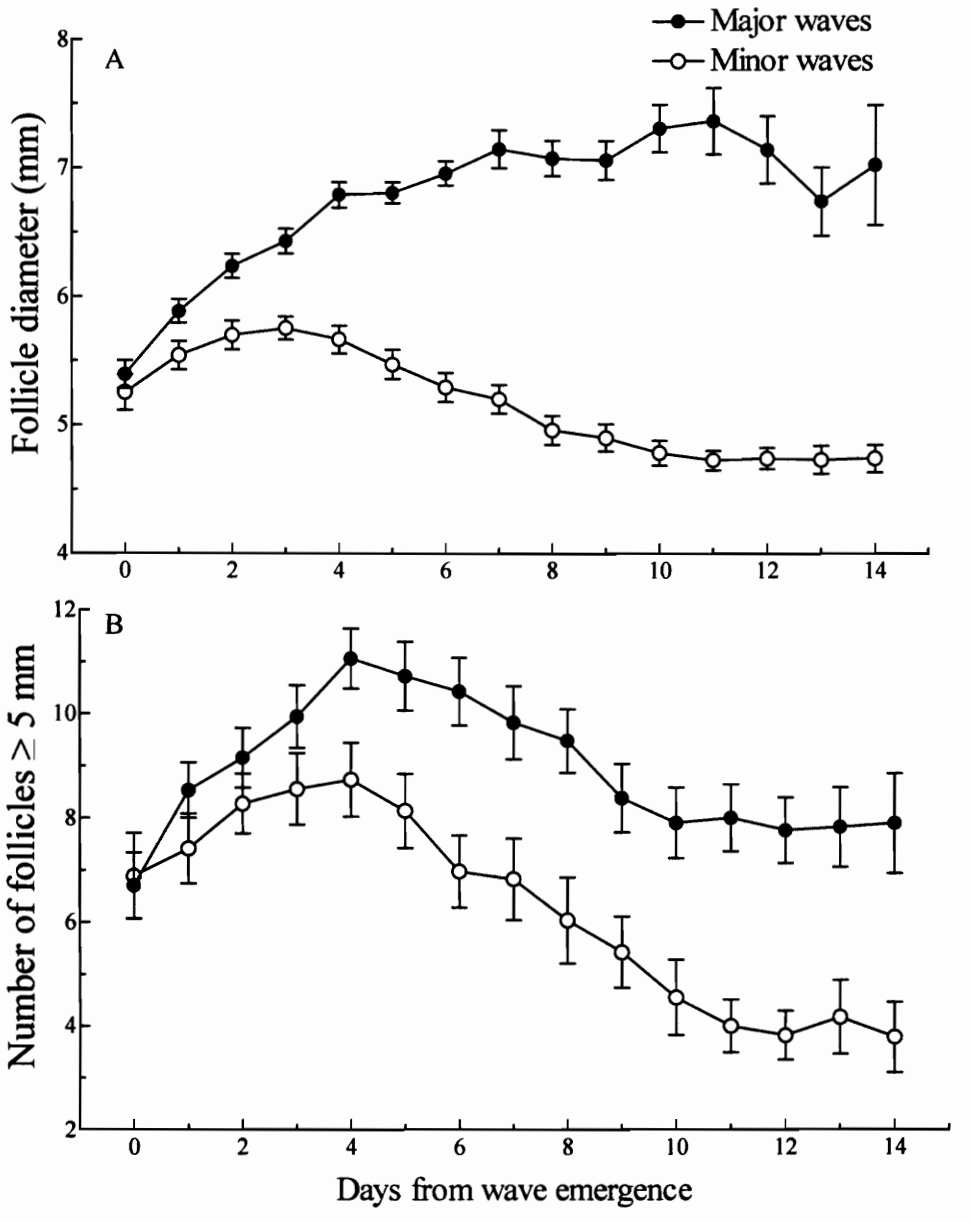

Figure 4.3: Mean diameter of all follicles ≥ 6 mm (A) and the number of follicles ≥ 5 mm (B) for major waves (●; n=67) and minor waves (○; n=52).

Day of selection, diameter of the dominant follicle at selection and diameter of the largest subordinate follicle at selection are shown in Table 4.4 (A,B). On the day of selection, the dominant follicle was 10 mm and the largest subordinate follicle was 8 mm in both major ovulatory and anovulatory waves ($p>0.05$ respectively). The dominant follicle became larger than the first and second subordinate follicles 3 days after wave emergence in both major ovulatory waves and major anovulatory waves, indicating that selection occurred at this time (Figure 4.4, $p<0.05$). The dominant follicle emerged 3 days later than the 1st and 2nd subordinate follicles in major ovulatory waves and 2 days later in major anovulatory waves.

Table 4.4: Selection of the dominant follicle (mean ± SEM) of major waves (i.e., ovulatory and anovulatory) in women with 2- and 3-wave patterns of follicle development during the menstrual cycle.

Pattern	n	Day of selection (day 0 =1st ovulation)	Diameter on day of selection (mm)	
			Dominant follicle	Largest subordinate follicle
2 Waves				
Wave 1	5	1.4±0.9	10.4+0.4[a]	8.4+0.3[a]
Wave 2	34	19.1+0.5	10.5+0.4[a]	7.9+0.5[a]
Overall	39	---	10.4±0.3	8.3±0.3
3 Waves				
Wave 1	3	4.0±0.6	9.0±1.0[c]	6.7±1.3[c]
Wave 2	6	17.0+0.6	10.0+0.7[c]	8.0+0.7[c]
Wave 3	16	21.6+0.7	9.9+0.5[c]	7.9+0.3[c]
Overall	25	---	9.8±0.4	7.8±0.3

[a,b] Within columns for 2 wave patterns, values with no common superscripts are different.
[c,d,e] Within columns for 3 wave patterns, values with no common superscripts are different.
Significance indicated at $p<0.05$.

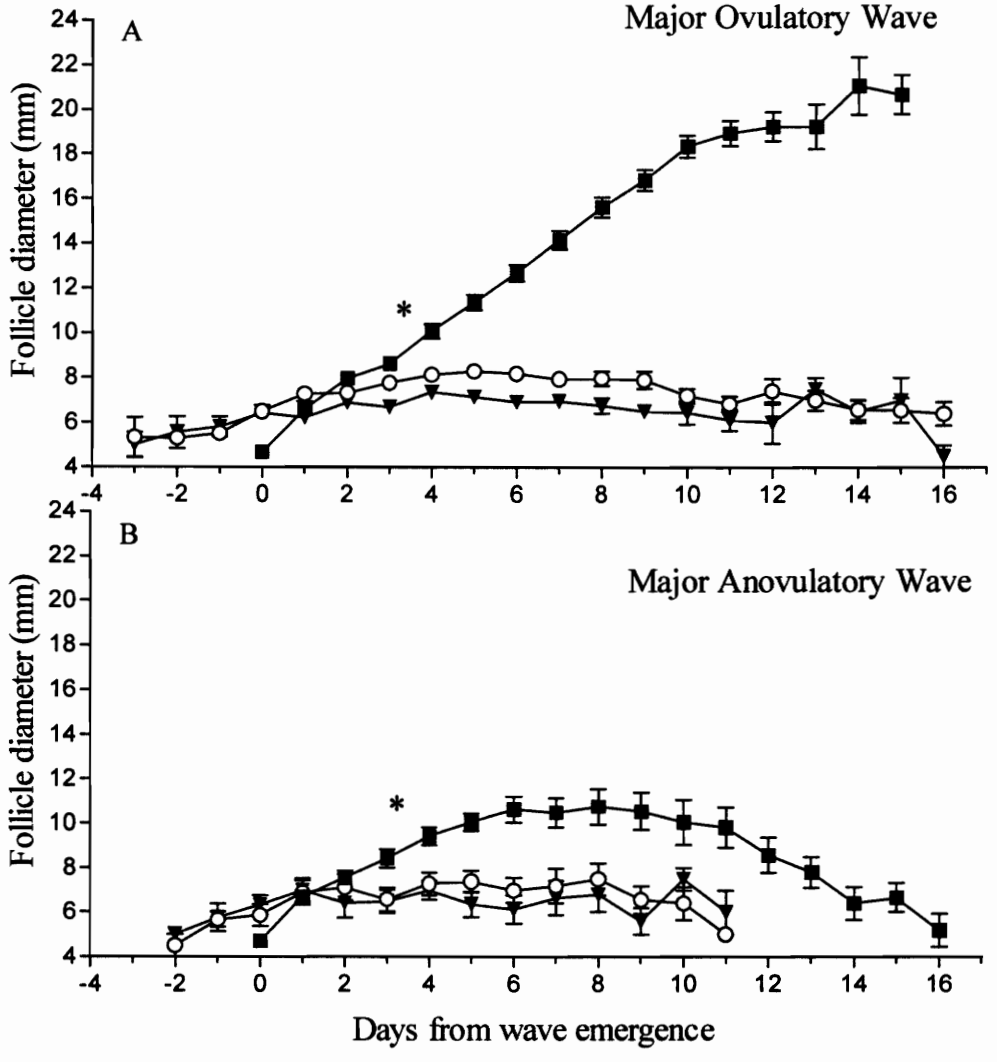

Figure 4.4: Diameter of the dominant (■), 1st subordinate (○) and 2nd subordinate (▼) follicles in major ovulatory waves (A; n=52) and major anovulatory waves (B; n=15). Asterisks indicate the first day at which a significant difference in diameter was detected.

Levels of FSH, LH and E_2 in women with 2 versus 3 follicular waves during the IOI are shown in Figure 5.5 (A,B,C). No differences in FSH and LH profiles between women with 2 versus 3 follicle waves were detected prior to the pre-ovulatory hormone surge (day effect: $p<0.0001$ respectively). However, estradiol levels increased earlier during the follicular phase (day effect: $p<0.0001$, wave effect: $p=0.30$, day*wave effect: $p=0.007$) in women with 2 versus 3 follicle waves. Similarly, the pre-ovulatory estradiol peak occurred 2 days earlier (i.e., 26 versus 28 days after ovulation) and the FSH and LH peaks occurred 1 day earlier (i.e., 27 versus 28 days after ovulation) in women with 2 wave cycles compared to those with 3 wave cycles. No differences in LH or estradiol concentrations were detected among women with major and minor patterns of follicle wave development (pattern effect: $p>0.05$; pattern*day effect: $p>0.05$).

A nadir in FSH was detected 3 days before the emergence of all anovulatory and ovulatory follicular waves (day effect: $p=0.004$, wave effect: $p=0.64$, day*wave effect: $p=0.06$) (Figure 4.6 A,B). The FSH nadir occurred 2 days earlier for major waves (i.e, day-3) compared to minor waves (i.e., day-1) (day effect: $p=0.008$, wave effect: $p<0.0001$, wave*day effect: $p=0.006$).

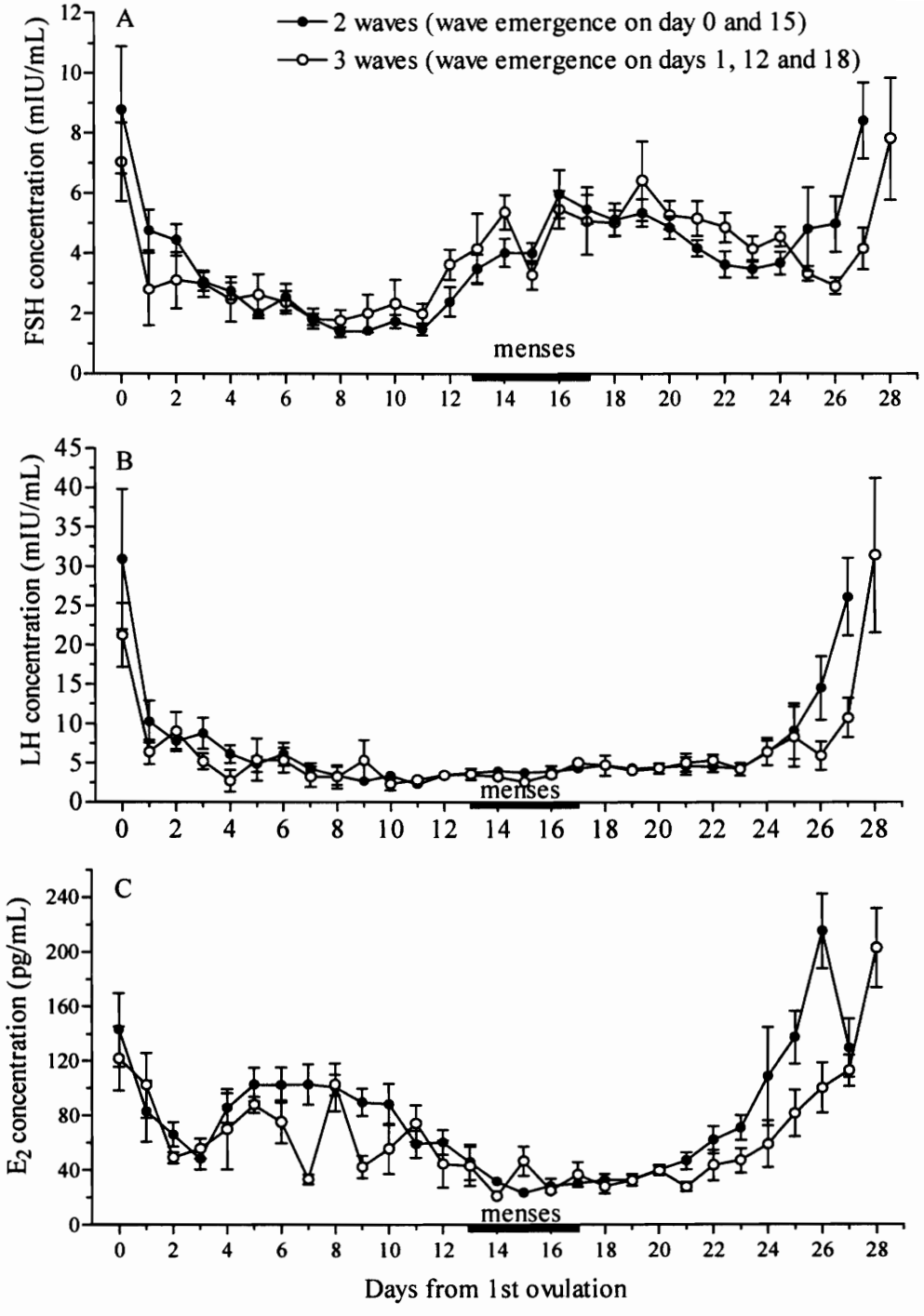

Figure 4.5: Serum FSH (A), LH (B) and estradiol-17β (C) concentrations during the interovulatory interval for women with 2 (●) and 3 (○) waves of follicle development. Endocrine data were centralized to the day of wave emergence and normalized to the mean IOIs of the respective wave patterns.

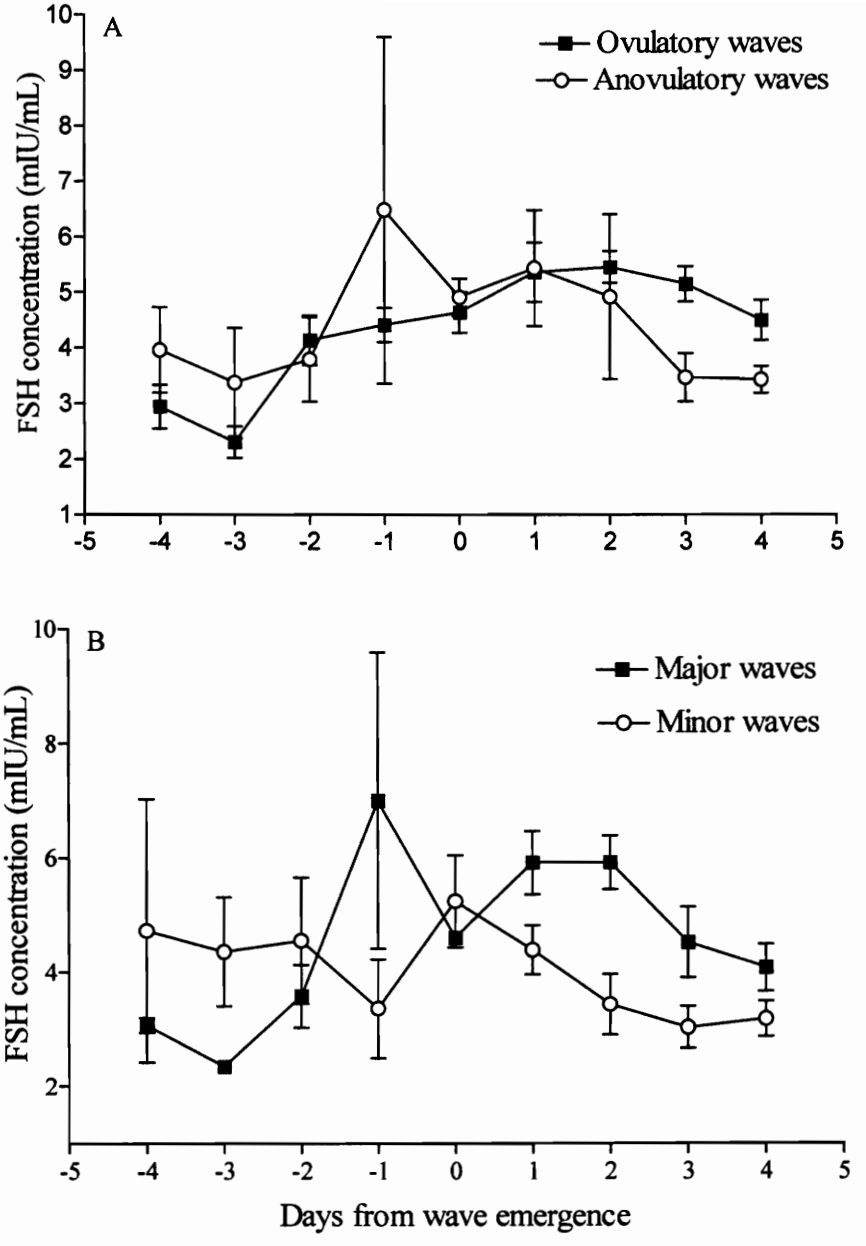

Figure 4.6: Serum FSH concentrations centralized to the day of wave emergence for (A) ovulatory (n=50; ○) and anovulatory (n=66; ■) waves and (B) major (n=67; ■) and minor waves (n=52;○).

4.5 Discussion

Our evaluation of the changes in follicle diameter and the number of follicles > 5mm during an interovulatory interval supported the hypothesis that women exhibit major and minor wave patterns of ovarian follicular development during the menstrual cycle. Major waves were those in which a single dominant follicle was selected to grow larger than all other subordinate follicles of the wave. Minor waves were those in which selection of a dominant follicle was not manifest. Anovulatory major waves were those in which the dominant follicle preferentially grew and then regressed. Ovulatory major waves were those in which the dominant follicle ovulated. In this regard, follicular dynamics in women is comparable to that described in the equine species [72].

In all 50 of the women evaluated, the final wave of the IOI was an ovulatory major wave, while preceding waves were either minor or major anovulatory waves. The dominant follicle of major ovulatory waves was larger than that in major anovulatory waves. Selection of the dominant follicle in both major anovulatory and ovulatory waves occurred at a diameter of approximately 10 mm, 3 days after wave emergence.

The dominant follicle of major waves emerged 2-3 days later than the 1st and 2nd subordinate follicles. These findings in women are inconsistent with observations in other species (i.e., bovine, equine) in which the dominant follicle emerged earlier than subordinate follicles [79]. It seems unlikely that emergence of the dominant follicle would differ between species given the similarity in follicular wave dynamics. We attribute this discrepancy to limitations inherent to current ultrasound imaging techniques. A human ovary contains, on average, more follicles $\geq$ 4 mm than a bovine ovary [64, 67]. The multitude of follicles visualized on each ovary on any given day during a woman's cycle made it difficult to maintain the day-to-day identities of 4-5 mm follicles. Follow-up studies in women should be performed with an emphasis on the emergence of 2-5 mm follicles.

Follicle Stimulating Hormone, LH and E_2 appear to be differentially involved in the regulation of ovarian follicular wave dynamics in women. The emergence of both major and minor follicular waves was preceded by an increase in FSH which occurred in concert with an increase in the number of follicles $\geq$ 5 mm. The nadir in FSH which

preceded wave emergence occurred 2 days earlier in women with major versus minor follicular waves.

Estradiol levels increased earlier during the follicular phase in women with 2 versus 3 follicular waves, presumably due to earlier emergence of the dominant follicle. Similarly, pre-ovulatory surges of FSH, LH and E_2 occurred earlier in women with 2 versus 3 follicle waves, resulting in a shorter IOI in women with 2 waves. We concluded that follicle development was the primary factor which determined the length of the menstrual cycle. In addition, it has previously been thought that the rise in circulating estradiol during the luteal phase of the menstrual cycle was due to the steroidogenic activity of the CL [83]. Although the CL does produce estradiol during the luteal phase, the levels observed in the present analysis are more consistent with the notion of a follicular, rather than luteal, origin of estradiol.

We were not able to identify differences in the concentrations of estradiol and LH in women exhibiting major versus minor patterns of follicle wave development. In retrospect, the every third day stratification scheme used for drawing blood was insufficient to allow finely detailed correlations between reproductive hormone levels and the wave-like nature of follicular development we observed. These findings, taken together with observations from a recent study [84], indicate that more frequent (eg., twice daily) venipuncture is necessary to quantify the precise changes in circulating concentrations of gonadotropins and steroid hormones during a woman's cycle and relate them to specific patterns of follicular growth. However, this approach is considered unfavorably by most research volunteers.

Human follicular development, in its entirety, occurs from a diameter of approximately 0.03 mm and continues for approximately 12 menstrual cycles until ovulation is achieved [4]. We were able to examine follicles at only advanced stages of development (i.e., $\geq$ 4 mm), over the course of only one cycle. It appeared, although we were not able to quantify, that follicular development in women may occur as a hierarchy. Follicles $\geq$ 4 mm grew in minor and major waves of development while smaller follicles (i.e., < 4 mm) appeared to grow and regress in a random fashion during the IOI. The growth dynamics of follicles < 4 mm in women are not known. Future work in this area will provide valuable insight into understanding the mechanisms of

follicle recruitment, selection and endocrine regulation of follicular growth and atresia. We expect that the use of 3D ultrasonographic imaging technologies will allow us to identify precise changes in the location and morphology of follicles and corpora lutea in the ovary, not currently possible using 2D imaging techniques. Future studies should also evaluate whether luteal progesterone plays a role in regulating the development of major and minor follicular waves in women.

The discovery that major and minor waves of follicle development occur during the menstrual cycle provides a new model for understanding human ovarian follicular development. Selection of a dominant follicle for preferential growth and development to an ostensibly ovulatory diameter can occur at more than one time during the menstrual cycle. We anticipate that the knowledge of major and minor follicular waves in women will have profound implications for the study, diagnosis and treatment of female infertility (i.e., ovarian stimulation for ovulation induction and in vitro fertilization) and the development of safer and more efficacious hormonal contraceptive formulations.

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Chapter 5

DOES THE CORPUS LUTEUM INFLUENCE THE DEVELOPMENT OF OVARIAN FOLLICULAR WAVES IN WOMEN?

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5.1 Abstract

Objectives: Waves of ovarian follicular development during the human menstrual cycle have recently been documented in our laboratory. Our objective was to ultrasonographically evaluate the corpus luteum (CL) to test the hypothesis that the CL would influence the development of ovarian follicular waves in women.

Materials and Methods: Fifty women underwent daily transvaginal ultrasonography to monitor ovarian status for one interovulatory interval (IOI). Ultrasonographic images of the CL were obtained at each visit and measurements of luteal area and numerical pixel value (NPV) were tabulated. Blood samples were drawn every third day during the IOI to determine serum progesterone concentrations.

Results: No differences were detected in the length of the luteal phase or lifespan of the CL between women with 2 and 3 waves of follicular development. The CL began to regress 7 days after ovulation (i.e., before the emergence of the second wave) in women with 2 and 3 follicle waves. No differences in maximal luteal area or progesterone concentration were detected. However, luteal area was greater in the follicular phase in women with 2 versus 3 follicle waves. Serum progesterone was greater in the luteal phase in women with 2 versus 3 follicular waves. Luteal area (follicular phase) and serum progesterone levels (luteal phase) were greater in women with minor minor major versus major major major wave patterns. No differences in luteal NPV were detected between women with 2 versus 3 follicle waves or among women with different follicle wave patterns.

Conclusions: Our hypothesis that the CL influenced the development of ovarian follicular waves in women was supported. We interpreted our results to mean that the CL exerted a suppressive effect on the emergence and development of follicular waves during the human menstrual cycle. These findings were not comparable to those previously documented in the bovine species. Further studies are required to determine whether the CL acts in a species-specific manner to influence ovarian follicle wave dynamics.

5.2 Introduction

The corpus luteum (CL) is a dynamic endocrine gland, believed to be responsible for regulating ovarian follicular development during the human menstrual cycle. Over the past 50 years, it has become widely accepted that antral follicular development $\geq$ 2 mm begins in the late luteal phase of the menstrual cycle, continues during the follicular phase and culminates in ovulation at mid-cycle [1-3]. Follicular quiescence has been thought to occur in the luteal phase of the cycle, due to the inhibitory effects of luteal progesterone on pituitary gonadotropin secretion [4-7]. It has also been speculated that the secretion of inhibin from the CL may elicit a negative effect on FSH secretion and follicle development during the luteal phase [8-10]. Late antral follicle development during the luteal phase has been reported occasionally [3, 11, 12], but it has been unexplained or has appeared to manifest an abnormal menstrual cycle.

We have recently documented waves of antral follicular development during the human menstrual cycle [13], contrary to the previously held notions of folliculogenesis in women. Sixty-eight percent of 50 women evaluated exhibited 2 waves of follicle development, while the remaining 32% exhibited 3 waves. We further characterized follicle waves into major and minor patterns [14]. Major waves were those in which one follicle became dominant over all other follicles in the wave. Minor waves were those in which dominance was not manifest. Fifty-eight percent of women exhibited a minor major wave follicle pattern (- +), 10% exhibited a major major pattern (+ +), 20% exhibited a minor minor major pattern (- - +), 6% exhibited a minor major major pattern (- + +) and 6% exhibited a major major major wave pattern (+ + +) [14]. The emergence of each follicle wave appeared to be preceded by a rise in circulating concentrations of FSH [14]. The ovarian follicular wave phenomenon has provided a new model for the study of human ovarian folliculogenesis. The precise mechanisms underlying the development of ovarian follicular waves in women, however, have not yet been determined.

Ovarian follicular waves in women are comparable to those previously described in domestic animals (i.e., bovine and equine models) [15-17]. The similarities in follicle growth dynamics between species has led to the use of the bovine

model for the study of human ovarian function [18]. It has been documented in the bovine species that regression of anovulatory follicular waves during the estrous cycle occurred through negative feedback effects of luteal progesterone on LH pulse frequency and estradiol production [19, 20]. Periodic development of anovulatory follicle waves continued during the bovine estrous cycle until the CL regressed [21]. Luteal regression occurred later in animals with 3 versus 2 follicle waves [21, 22], and progesterone levels remained elevated longer in 3 versus 2 wave animals [23]. In both 2 and 3 wave animals, the CL regressed after emergence of the ovulatory wave, and before the emergence of the subsequent wave. The emergence of a third wave was associated with a longer luteal phase, and the viable dominant follicle present at the time of luteolysis became the ovualtory follicle [21]. Ultrasonographic image attributes of the bovine CL have been evaluated. Mean numerical pixel value (NPV) of the CL decreased during the growth phase and increased during luteal regression [24]. Ultrasound image attributes of the bovine CL reflected luteal and plasma progesterone content, and histomorphological characteristics of the CL [25].

The objective of the present study was to evaluate the CL to test the hypothesis that the CL influences the development of ovarian follicular waves during the human menstrual cycle. Specifically, we hypothesized that differences in luteal function (as reflected by changes in luteal area, NPV and serum progesterone levels) would be detected between women with 2 versus 3 waves of follicle development and among women with major and minor patterns of follicle growth.

5.3 Materials and Methods

Fifty women participated in a study to characterize ovarian follicular wave dynamics during the menstrual cycle [13]. Luteal imaging data and serum progesterone levels collected from these 50 women were used in the present study to evaluate the influence of the CL on follicle wave dynamics. Participants were assessed, by history and physical examination, to be healthy women of reproductive age (mean age $\pm$ SD = 28.0 $\pm$ 6.9 years, range = 19 - 43 years). Women who were currently pregnant or had been pregnant or lactating 6 months prior to initiating study procedures, had used hormonal contraception 3 months before enrolling, had a history of irregular menstrual

cycles, were taking medication(s) known or suspected to interfere with reproductive function, or were planning surgery during the study period were not eligible to participate. The study protocol was approved by the Institutional Review Board of the University of Saskatchewan. Informed consent was obtained from all participants before initiating study procedures.

Each participant underwent daily transvaginal ultrasonographic evaluation of her ovarian status for one interovulatory interval (IOI). An IOI was defined as the interval from one ovulation to the subsequent ovulation. Scans were initiated 12 days after menses (i.e., before the first ovulation) and were continued until 3 days after the second ovulation. Ovulation was defined as the disappearance of a large follicle (>15 mm) that had been identified by ultrasonography on the previous day, and was confirmed by the subsequent visualization of a corpus luteum. Corpora lutea were characterized ultrasonographically by a thickened wall with or without a hypoechoic central fluid-filled area [26]. High-resolution Ultramark 9 and ATL HDI 5000 ultrasound machines with 5-9 MHz multi-frequency convex array transducers were used to image the CL. Ultrasonographic examinations were performed by a single sonographer (ARB) approximately 90% of the time. A second sonographer (RAP) was available when the primary sonographer was not present.

Daily ultrasonographic images of the CL were obtained at each examination. A customized computer program designed in our laboratory (Synergyne©, Women's Health Imaging Research Laboratory, Saskatoon, SK) was used to tabulate the cross-sectional area of the CL (based on the shape of an ellipse) and numerical pixel value (NPV). Cross-sectional area of the CL was determined by outlining the external border of the CL and the internal border of the central fluid-filled cavity (if present). The area of the central fluid-filled cavity (if present) was subtracted from the overall luteal area to give the area which represented luteal tissue.

Numerical pixel value is a quantitative measurement of the intensity of the pixel elements that comprise an ultrasound image. Numerical pixel value was quantified using values ranging from 0 (black) to 256 (white). The CL image was divided into 4 equal quadrants, and a circular region was used to sample NPV from each quadrant. Sample regions encompassed only luteal tissue, avoiding the ovarian stroma and fluid-

filled areas in the CL. The mean NPV was calculated by averaging the NPV measurements from the 4 quadrants.

Blood samples were drawn to determine serum progesterone concentrations every third day in a stratified manner among women so that each day of the IOI was represented. Blood was collected into a 7 mL clot-activated tube and allowed to sit at room temperature for 15-30 minutes. Blood was then centrifuged for 10 minutes at 700G and the serum was drawn off and stored frozen at -20°C. Sequential competitive fluorescence immunoassays (Immulite®) were performed to measure serum progesterone levels. The inter-assay co-efficients of variation were as follows: low=10.8%, medium=7.0% and high=10.8%. The minimal detectable limit was 0.2 ng/mL.

Luteal area and NPV data were normalized to the mean IOI for women with 2 follicle waves or 3 follicle waves. Luteal data were further normalized to the respective mean IOI for women with major and minor patterns of follicular wave dynamics. Endocrine data were normalized to the mean IOI and centralized to the mean day of emergence of each wave. Repeated measures analyses were performed to assess changes in luteal area, NPV and progesterone concentration during the IOI (PROC MIXED, SAS/STAT Software, 2002). Independent sample t-tests and Analyses of Variance using Scheffe's Post Hoc Tests (SPSS Version 11, 2002) were used to compare maximum luteal area, NPV and progesterone concentrations between women with 2 versus 3 follicle waves and among women with different major and minor wave patterns.

5.4 Results

Serial ultrasonographic images of the CL during development and regression in one research volunteer are shown in Figure 5.1 (A-E). Corpora lutea were of 2 morphological types: those with a central fluid-filled cavity and those without. A fluid-filled cavity was visualized in the CL of 39/50 women evaluated (78%), while a fluid-filled cavity was not visualized in the remaining 11/50 women (22%). Ultrasonographic images of corpora lutea with and without central fluid-filled cavities are shown in Figure 5.2 (A-D).

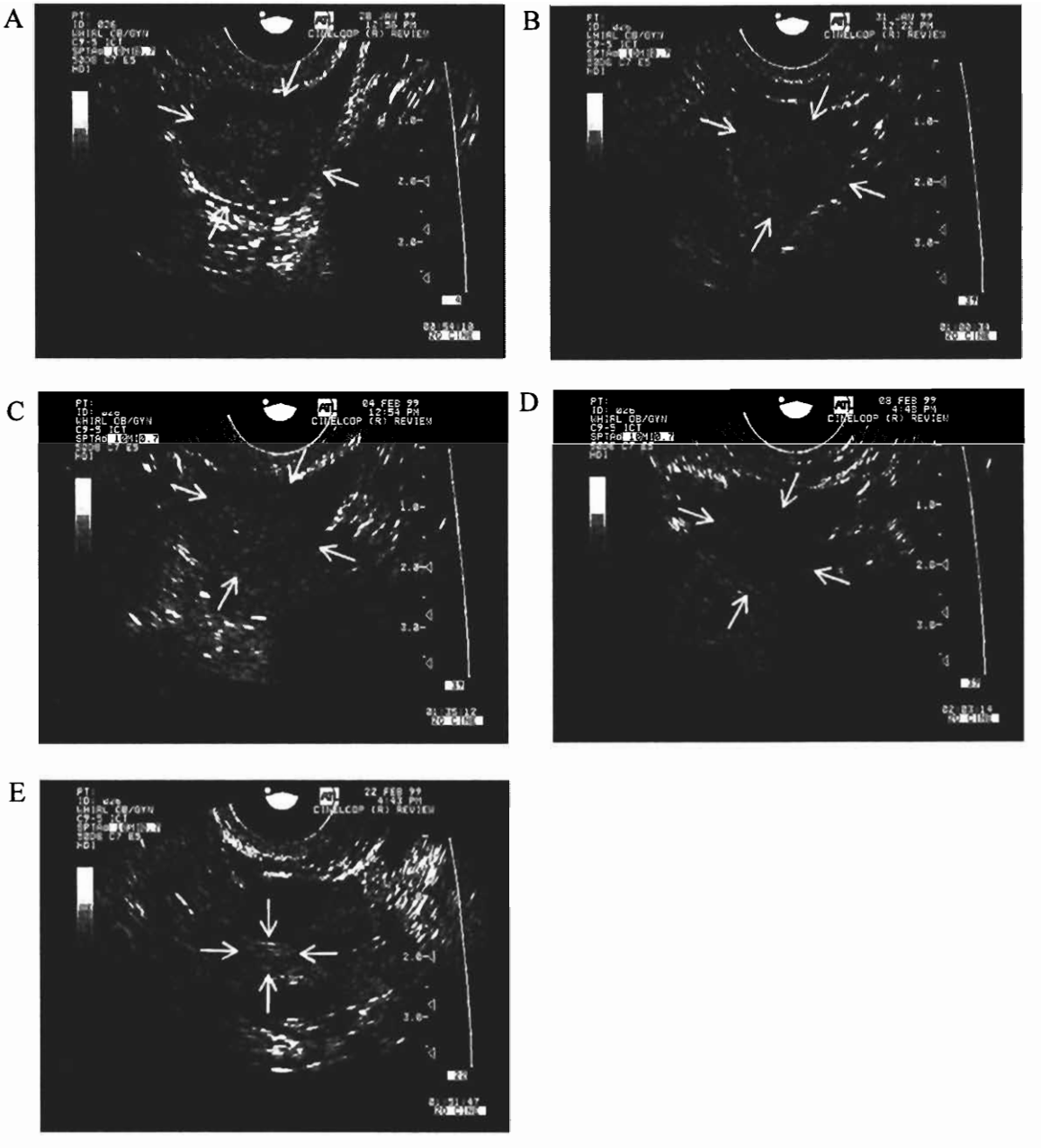

Figure 5.1: Ultrasonographic images of the corpus lutem (CL) during development and regression in one woman. Arrows delineate the outer borders of the CL. Images were obtained 3 (A), 7 (B), 11 (C), 14 (D) and 25 (E) days after ovulation.

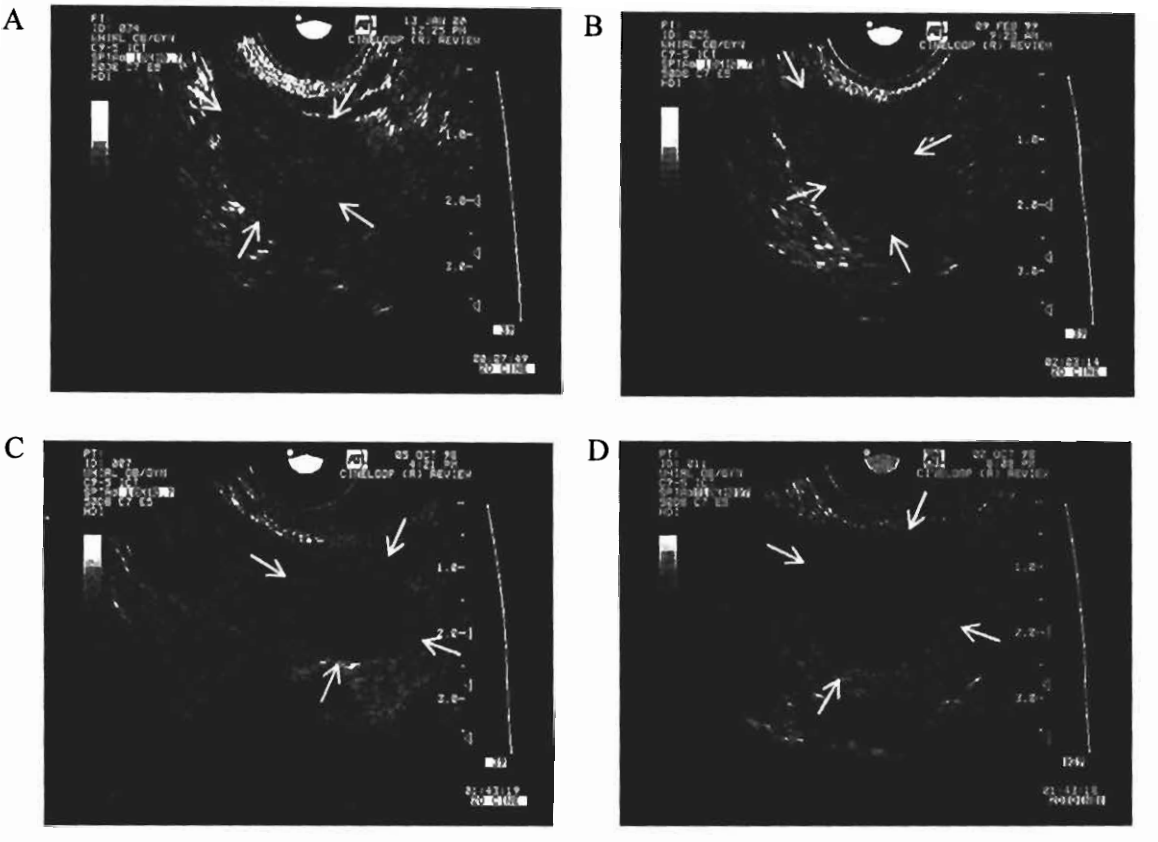

Figure 5.2: Ultrasonographic images of corpora lutea with no central fluid-filled cavities (A) and small (B), medium (C) and large (D) fluid-filled cavities. Arrows delineate the outer borders of the CL.

No differences were detected in the mean luteal lifespan of women with 2 follicle waves (19.6 ± 0.8 days) versus 3 follicle waves (19.9 ± 0.7 days, p=0.4). Likewise, the length of the luteal phase was not different between women with 2 (13.4 ± 0.2 days) and 3 waves of follicle growth (13.1 ± 0.4 days, p=0.60).

The day of luteal regression was determined by the first detectable decrease in luteal area during the IOI. Luteal regression occurred 7 days after ovulation (i.e., before emergence of the second wave) in women with both 2 and 3 waves of follicle growth (Figure 5.3 A,B). Therefore, the dominant follicle present at the time of luteolysis did not become the dominant ovulatory follicle.

No differences in maximum luteal area and peak progesterone concentrations were detected between women with 2 versus 3 follicular waves (Table 5.1, p>0.05). In comparison, the days on which maximum luteal area, peak progesterone concentration and luteal regression (as determined by the first decrease in luteal area) were obtained were not different between groups (Table 5.1, p>0.05).

Table 5.1: Comparisons of maximum luteal area and progesterone concentrations between women with 2 and 3 waves of follicular development. Day of luteal regression was determined by the first detectable decrease in luteal area during the IOI. Day 0 = day of 1st ovulation.

	2 Waves	3 Waves	P-Value
Maximum Luteal Area (mm^2)	307.2 ± 13.3	337.1 ± 27.7	0.27
Day of Maximum Luteal Area	6.1 ± 0.3	5.5 ± 0.5	0.33
Day of Luteal Regression	7.3 ± 0.3	6.9 ± 0.5	0.59
Maximum Progesterone (ng/mL)	14.3 ± 0.9	11.9 ± 1.1	0.14
Day of Maximum Progesterone	5.8 ± 0.3	6.0 ± 0.4	0.66

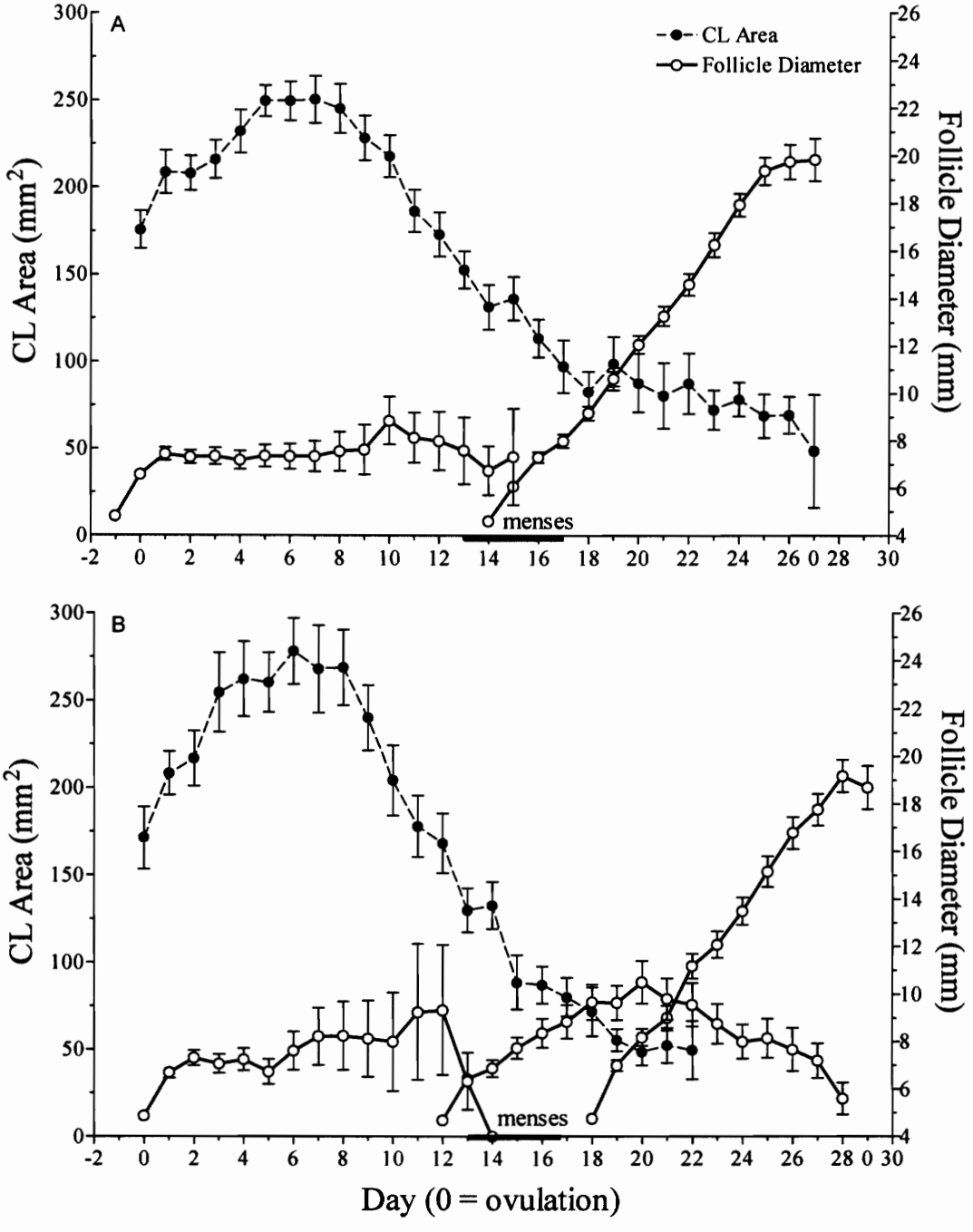

Figure 5.3: Cross-sectional area of the CL (●; mm^2) and diameter of the largest follicle of each wave (○; mm) in women with 2 (A) and 3 (B) waves of follicle development during the IOI.

Luteal area during the IOI in women with 2 versus 3 waves and in women with different wave patterns are shown in Figure 5.4 (A-C). Luteal area was not different during the luteal phase (i.e., days 0-12) in women with 2 versus 3 follicular waves (day effect: $p<0.0001$, wave effect: $p= 0.38$, day*wave effect: $p=0.73$). However, luteal area was greater during the follicular phase (i.e., days 13-22) in women with 2 versus 3 follicle waves (day effect: $p<0.0001$, day*wave effect: $p=0.04$). In women with 2 follicle waves, no differences in luteal area during the IOI were detected between women with - + and + + wave patterns (day effect: $p<0.0001$, pattern effect: $p=0.96$, day*pattern effect: $p=0.69$). However, in women with 3 follicle waves, luteal area was slightly greater during the follicular phase for those with - - + versus + + + wave patterns (day effect: $p<0.0001$, pattern effect: $p=0.04$, day*pattern effect: $p=0.98$).

Luteal area was highly correlated with serum progesterone levels in women with 2 ($r=+0.88$) and 3 ($r=+0.90$) waves of follicle development. Serum progesterone concentrations in women with 2 and 3 follicle waves and major and minor patterns of follicle growth are shown in Figure 5.5 (A-C).

Progesterone was greater on day 4 of the luteal phase in women with 2 waves compared to women with 3 waves (day effect: $p<0.0001$, wave effect: $p=0.04$, day*wave effect: $p= 0.58$). In women with 2 follicle waves, progesterone during the luteal phase was greater in women with + + versus - + wave patterns (day effect: $p<0.0001$, pattern effect: $p=0.25$, day*pattern effect: $p=0.002$). In women with 3 follicle waves, progesterone concentrations were greater in women with - - + versus +++ wave patterns (day effect: $p<0.0001$, pattern effect: $p=0.60$, day*pattern effect: $p=0.002$).

Luteal NPV during the IOI decreased during the luteal phase (day 0-11), followed by an increase in the follicular phase (day 11-16) (day effect: $p<0.0001$). However, no differences in luteal NPV were detected between women with 2 versus 3 follicle waves or among women with different wave patterns (day effect: $p<0.0001$, wave effect: $p>0.05$, day*wave effect: $p> 0.05$, Figure 5.6).

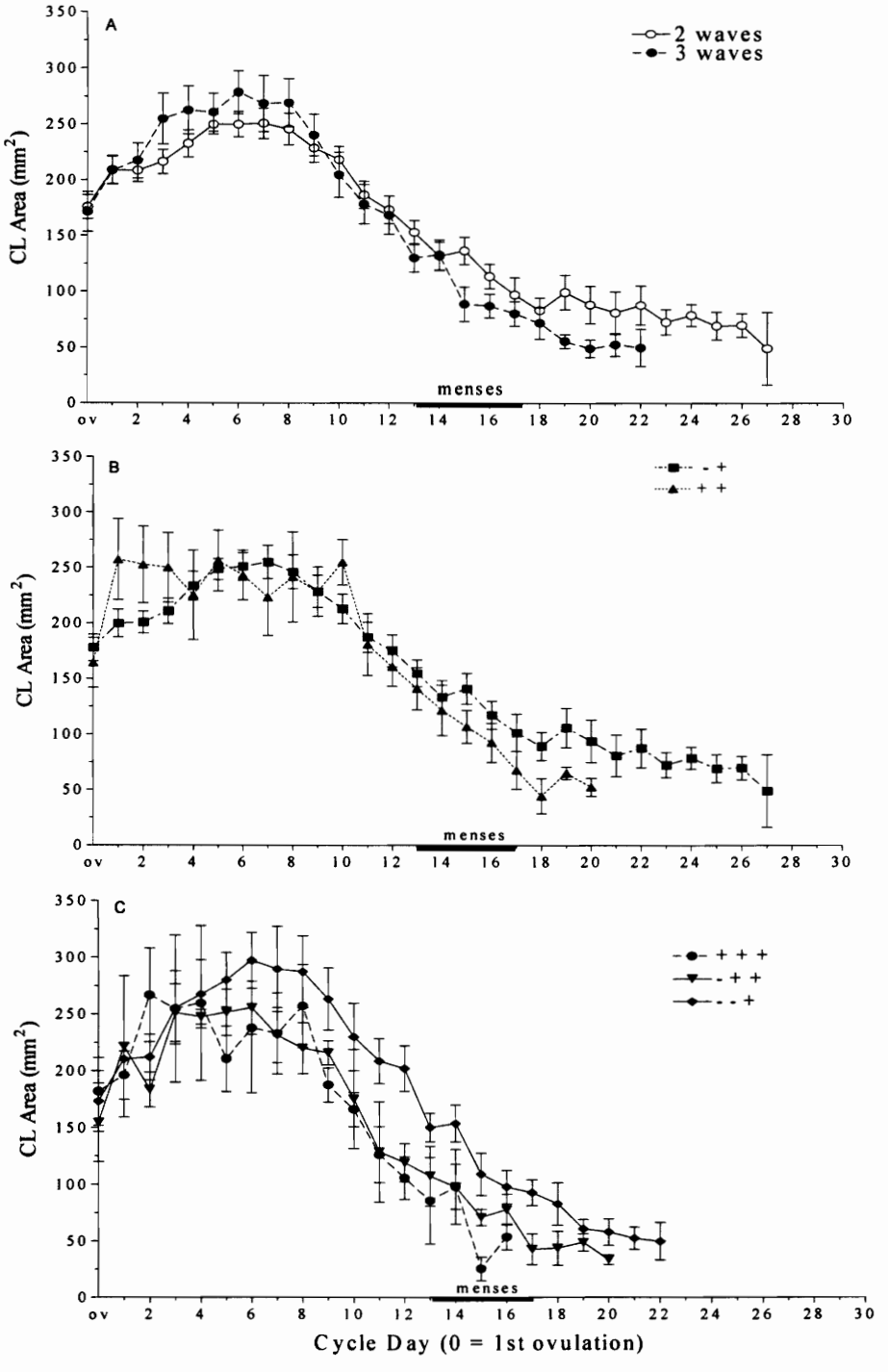

Figure 5.4: Cross-sectional area of the CL (mm^2) for women with (A) 2 waves (n=34; ○) and 3 waves (n=16; ●) of follicle development during the IOI. In women with 2 follicle waves (B), luteal area is compared between women with - + (n=29; ■) and + + (n=5;▲) wave patterns of follicle development. In women with 3 follicle waves (C), luteal area is compared between women with - - + (n=10; ♦), - + + (n=3;▼) and + + + (n=3; ●) wave patterns of follicle development.

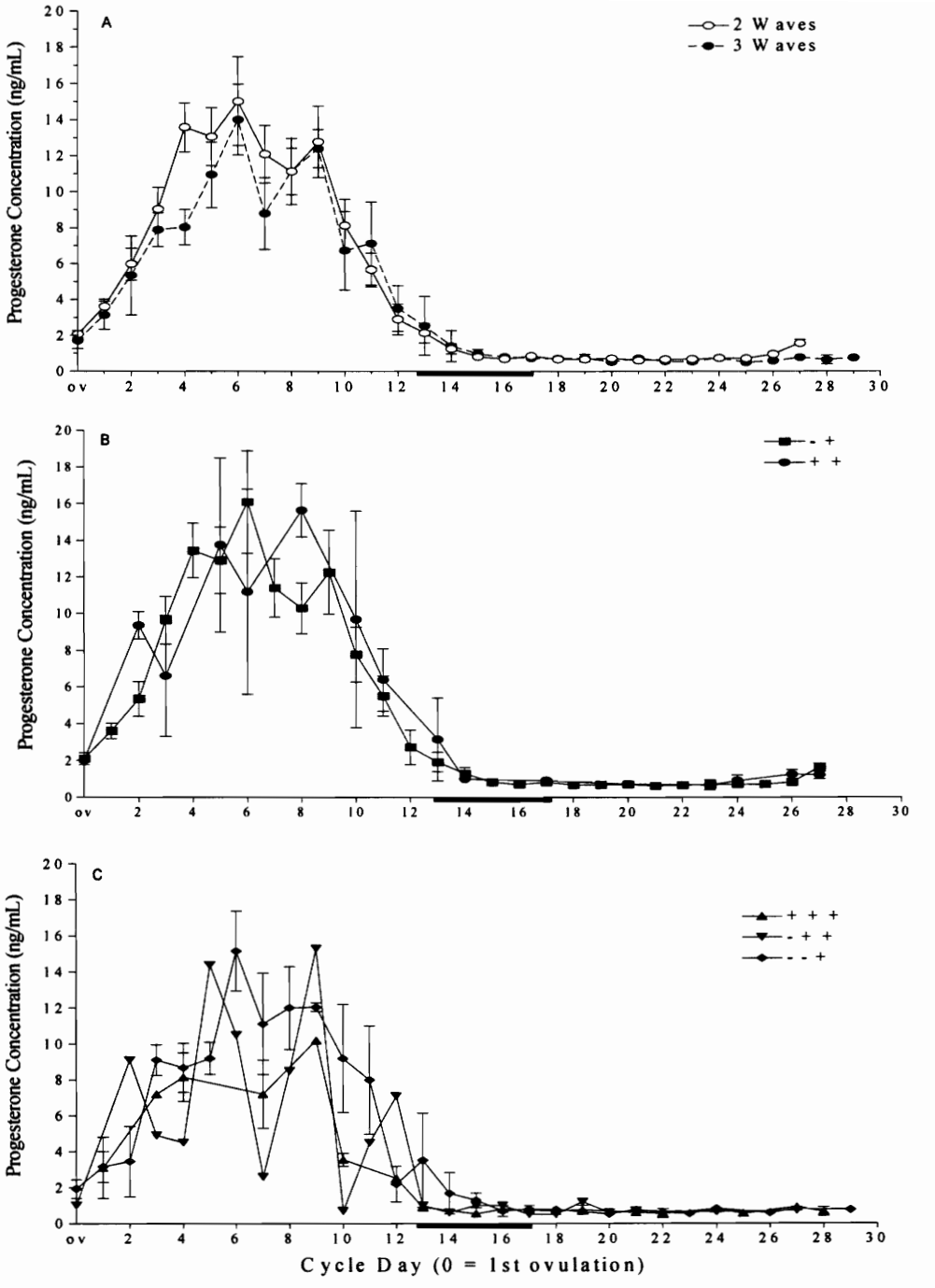

Figure 5.5: Serum progesterone concentrations (ng/mL) for women with (A) 2 waves (n=34; ○) and 3 waves (n=16; ●) of follicle development during the IOI. In women with 2 follicle waves (B), progesterone levels are compared between women with - + (n=29; ■) and + + (n=5; ●) wave patterns of follicle development. In women with 3 follicle waves (C), progesterone levels are compared between women with - - + (n=10; ♦), - + + (n=3; ▼) and + + + (n=3; ▲) wave patterns of follicle development.

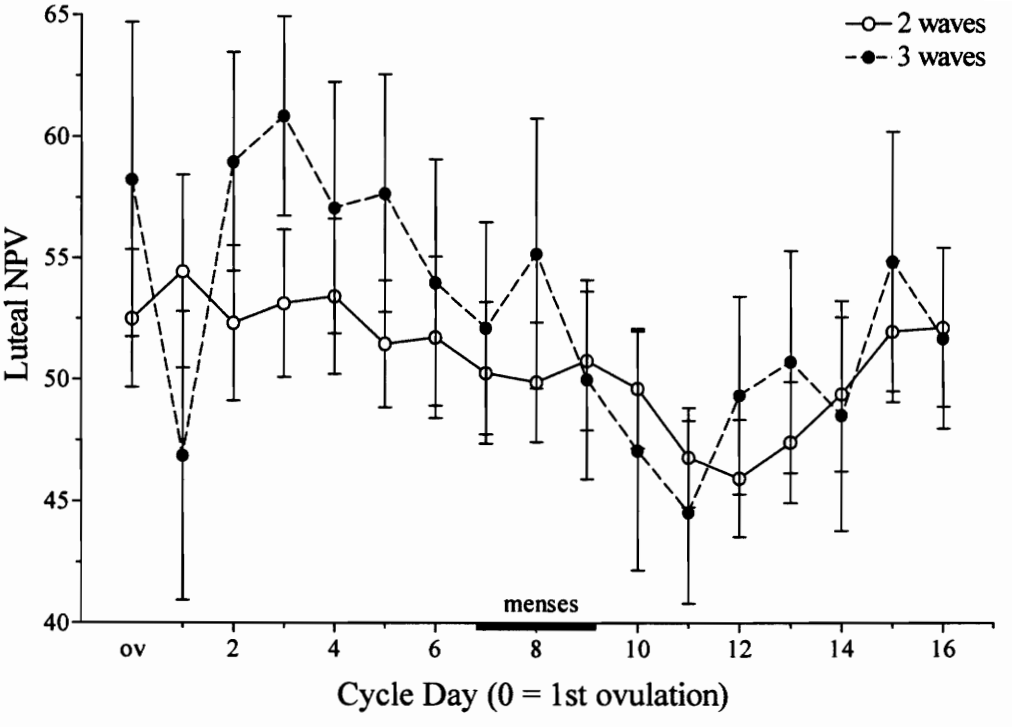

Figure 5.6: Numerical Pixel Values (NPV) of ultrasonographic images of the CL in women with 2 (n=34; ○) and 3 (n=16; ●) waves of follicle development.

5.5 Discussion

Our hypothesis that differences in luteal area and progesterone would be detected between women with 2 versus 3 follicle waves, and among women with major and minor follicle wave patterns, was supported. No differences in luteal phase length, maximal luteal area or peak progesterone concentration were observed in women with 2 and 3 follicle waves. However, the CL produced more progesterone during the luteal phase and remained larger during the follicular phase in women with 2 versus 3 follicle waves. We interpreted our results to mean that the CL exerted a suppressive effect on the emergence of a third wave in women with 2 follicle waves. Luteal regression in women, as determined by the first decrease in luteal area, occurred at the midpoint of the first follicle wave in women with both 2 and 3 follicle waves. That is, the second ovulatory wave emerged after luteal regression in women with 2 follicle waves. Similarly, the second anovulatory wave and third ovulatory wave emerged after luteal regression in women with 3 follicle waves. Therefore, waves of anovulatory follicular development did not continue until luteal regression, and the follicle present at the time of luteolysis did not become the dominant ovulatory follicle. These findings are completely opposite to what has been documented in the bovine species [21].

The regression of anovulatory dominant follicles during the bovine estrous cycle has been shown to occur through negative feedback effects of luteal progesterone on LH pulse frequency [19, 20]. Periodic development of anovulatory follicle waves during the bovine estrous cycle continued the until the CL regressed [21]. In both 2 and 3 wave animals, the CL regressed after emergence of the ovulatory wave, and before the emergence of the subsequent wave. The emergence of a third wave was associated with a longer luteal phase, and the viable dominant follicle present at the time of luteolysis became the ovualtory follicle [21]. Luteal regression occurred later in animals with 3 versus 2 follicle waves [21, 22], and progesterone levels remained elevated longer in 3 versus 2 wave animals [23]. Progesterone levels continued to rise during the bovine estrous cycle until the late follicular phase, at which time they dropped dramatically and the dominant follicle present ovulated [20]. This did not appear to be the case in women, as the CL regressed and progesterone concentrations decreased much earlier, in the mid-luteal phase of the menstrual cycle.

We reported greater progesterone concentrations in the luteal phase and luteal area in the follicular phase in women with - - + versus + + + wave patterns of follicle growth. These findings suggested a suppressive effect of the CL on follicle selection. Higher progesterone levels followed by greater luteal area was associated with the development of minor anovulatory waves (i.e., waves in which selection of a dominant follicle was not manifest). Our results lend credence to the notion that elevated luteal progesterone production prevented selection of a dominant follicle in minor anovulatory waves that developed prior to the ovulatory wave. The finding that progesterone concentrations were higher during the luteal phase in women with ++ versus - + follicle waves did not compare with the above mentioned suppressive effect of luteal progesterone on follicle selection. We concluded that the latter findings were inconclusive, and may have been associated with measurement error.

The differences observed in the growth and regression of the CL during the human menstrual cycle compared to that previously documented in the bovine estrous cycle could be due to 3 factors. It is possible that the CL may act in a species-specific manner to influence the development of ovarian follicular waves. Major and minor waves of follicle development occur during the human menstrual cycle, but not during the bovine estrous cycles. This factor alone, could be responsible for a differential role of the CL in regulating follicle wave dynamics. Major and minor wave patterns have been observed in the equine species [17]. The equine species therefore may be a more appropriate model for studying ovarian follicular wave dynamics in women. The amount of research performed in the equine species, however, is limited, and the role of luteal progesterone in regulating major and minor follicle wave dynamics in mares has not yet been elucidated. It is plausible that we have not yet been able to detect the precise mechanisms by which the CL regulates follicular growth and regression. The number of women exhibiting + + (n=5/50), - + + (n=3/50), and + + + (n=3/50) patterns of follicle wave dynamics were small. Small sample sizes and the inability to obtain frequent (i.e., twice daily) blood samples from women in the study, may have led to difficulty in detecting precise changes in luteal progesterone production during the IOI. It would also be useful to evaluate the differences in luteal phase estradiol and inhibin concentrations between women with 2 and 3 follicle waves and different patterns of

major and minor follicle wave dynamics. It is possible that estradiol and/or inhibin may act to inhibit FSH secretion during the luteal phase. Decreased FSH concentrations may be associated with inhibiting selection in women with minor anovulatory waves and ovulation in women with major anovulatory waves during the luteal phase.

The hypothesis that differences in ultrasonographic image attributes of the CL would be detected in women with 2 and 3 waves, and among women with different follicle wave patterns, was not supported. Changes in luteal NPV were detected during the IOI, irrespective of follicle wave status. A decrease in luteal NPV occurred during luteal development in association with an increase in luteal area and progesterone concentrations. The subsequent increase in NPV during luteal regression occurred in association with a decrease in luteal area and progesterone concentrations. We interpreted these findings to mean that quantitative changes in luteal echotexture were reflective of changes in the physiologic status of the CL, as previously documented in the bovine species [24, 25]. Increased vascularization of luteal tissue in the bovine CL was associated with decreased NPV during luteinization (i.e., representative of decreased tissue density). Decreased vascularization and replacement of luteal tissue with fibrous connective tissue occurred in association with increased NPV during luteolysis (i.e., reflective of increased tissue density). No differences in luteal NPV were detected between women with 2 versus 3 follicle waves, or among women with different wave patterns of follicle development. The inability to detect differences among women with different numbers and patterns of follicle waves may have due to the small numbers of women occupying the ++, - ++ and +++ groups.

The wave phenomenon of human follicular development has provided a new model for studying human ovarian function. This model provides rationale for the notion that follicular development may be the primary determinant of menstrual cycle length. Earlier emergence of the dominant follicle in women with 2 versus 3 follicle waves was associated with earlier pre-ovulatory surges of LH, FSH and estradiol and a corresponding shorter IOI [13, 14]. Luteal progesterone has been believed to suppress the development of large antral follicles during the luteal phase of the menstrual cycle [4-7]. However, it is now known that antral follicle development occurs during the luteal phase in healthy women of reproductive age. Luteal progesterone appeared to

have a negative effect on the number of anovulatory waves that developed prior to the ovulatory wave. Higher luteal progesterone was associated with suppression of the maximum diameter attained by the leading follicles that developed during the luteal phase, thereby preventing follicle selection in minor anovulatory waves. Future studies should be performed to evaluate the influence of luteal phase estradiol and inhibin on follicle wave dynamics in women. Continued research should also focus on comparative aspects of luteal function in women and domestic animal species, particularly the equine species, in order to determine whether species-specific mechanisms exist to regulate the ovarian follicular wave phenomenon.

4.6 Acknowledgments

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Chapter 6

ENDOMETRIAL DEVELOPMENT IN ASSOCIATION WITH OVARIAN FOLLICULAR WAVES IN WOMEN

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6.1 Abstract

Objectives: To test the hypothesis that ultrasonographically detectable changes in the endometrium during the menstrual cycle would differ between women with 2 versus 3 waves of ovarian follicular development and among women with different major and minor wave patterns of follicle growth.

Materials and Methods: Fifty women of reproductive age (mean age $\pm$ SD=28.0 $\pm$ 6.9 years) underwent daily transvaginal ultrasonography for one interovulatory interval (IOI). Ultrasonographic images of the endometrium were obtained each day, and measurements of endometrial area and perimeter (based on the shape of an ellipse, in transverse plane) and thickness and pattern (in sagittal plane) were recorded. Endometrial endpoints were compared between women with 2 and 3 waves of follicle development and among women with different minor and major wave patterns of follicular growth during the IOI.

Results: Endometrial area, perimeter, thickness, and pattern increased earlier during the follicular phase in women with 2 versus 3 waves of follicular development ($p<0.05$). In women with 2 follicle waves, those with major major follicle wave patterns exhibited greater endometrial area and perimeter than women with minor major patterns ($p<0.05$).

Conclusions: Ultrasonographically-detectable changes in the endometrium occurred in association with follicle wave dynamics in women. Earlier development of the endometrium during the follicular phase in women with 2 versus 3 follicle waves was attributed to an earlier increase in dominant follicle estradiol production.

6.2 Introduction

Endometrial development during the human menstrual cycle is closely associated with changes in ovarian function. Granulosa cells of developing ovarian follicles in the follicular phase of the cycle produce estradiol, which stimulates the development of the endometrial lining [1-3]. In the few days before ovulation, progesterone levels begin to rise [2]. The source of the pre-ovulatory rise in progesterone levels is not fully known, but is believed to be the thecal, granulosa or interstitial cells [2, 4-6]. After ovulation, progesterone produced by the corpus luteum is believed to maintain the estrogen-primed endometrium and stimulate endometrial

glandular development to provide an environment conducive to implantation [1-3]. Several growth factors have also been shown to regulate endometrial development (for example, prostaglandins, interleukins, insulin-like growth factors); however, their precise roles are not fully elucidated [7-11]. Communication between the ovaries and uterus is required for reproductive success. It is therefore plausible that abnormal signaling mechanisms between the ovary and uterus are associated with abnormal endometrial development, infertility and recurrent embryonic loss.

Transvaginal ultrasonography has become an invaluable tool for evaluating the endometrium during natural menstrual cycles and treatment for infertility [12-16]. Ultrasonographically-detectable changes in the endometrium occur throughout the menstrual cycle in association with changes in concentrations of serum estradiol and progesterone [17, 18]. The endometrium is comprised of 2 layers: the stratum basalis which lies next to the myometrium, and the stratum functionalis which lines the endometrial cavity [19]. The thickness of the endometrium and relative echotexture (i.e., reflectivity) of the stratum functionalis and basalis compared to the myometrium are measurements used to assess the endometrium ultrasonographically. Endometrial thickness is measured as the distance between the anterior stratum basalis and posterior stratum basalis layers in sagittal plane [20, 21]. Endometrial thickness has been reported to increase during the follicular phase of natural menstrual cycles, reach a peak prior to ovulation, plateau during the early luteal phase and then decline prior to menstruation [14-18]. The increase in endometrial thickness during the follicular phase is associated with an increase in serum estradiol levels [17, 18, 22].

The endometrium appears ultrasonographically as a thin, simple hyperechoic single stripe immediately following menses (A pattern). The statum functionalis and basalis layers can be visually differentiated as the endometrium develops during the mid-late follicular phase (B pattern). A pronounced triple-line echotextural pattern, reflective of the differentiation of the stratum basalis and functionalis layers, is observed in the peri-ovulatory period in association with rising estradiol levels (C pattern). The triple-line pattern disappears after ovulation. A more homogeneous hyperechoic endometrium is observed as endometrial glands branch and proliferate under the influence of luteal progesterone production in the secretory phase (D pattern).

Visualization of active menstrual flow is indicative of menses (M pattern) [14, 18, 20, 22-24].

Ultrasonographic assessment of the endometrium has been evaluated as a method for predicting success following controlled ovarian hyperstimulation and in vitro fertilization. In many reports, a thick endometrium and/or triple-line echogenic pattern of the endometrium was associated with favorable IVF outcomes [25-35]. In contrast, other researchers reported no associations between ultrasonographic appearance of the endometrium and success following assisted reproduction [13, 36-40], and recommended that further research be performed before any definitive conclusions are made.

Limited research has been performed to evaluate the endometrium ultrasonographically during spontaneous menstrual cycles. Studies performed insofar have involved the assessment of small numbers of women using transabdominal ultrasonography sometimes in combination with endometrial biopsy and/or histologic assessment [14-18]. Serial evaluations of the endometrium during the menstrual cycle using high-resolution transvaginal ultrasonography in large samples of women have not yet been performed. Clinical observations of healthy, reproductively-aged women have led us to believe that endometrial development varies among women (Baerwald and Pierson, unpublished data). It is plausible that variability in endometrial growth may be attributed to variability in ovarian follicular dynamics among women.

The current state of knowledge on endometrial growth during spontaneous menstrual cycles has been based on previously held notions that ovarian follicles developed to ostensibly ovulatory diameters only during the follicular phase, followed by follicular quiescence during the luteal phase [41-48]. However, it is now known that waves of ovarian follicular development occur during the menstrual cycle [49]-[50]. Sixty-eight percent of 50 women evaluated exhibited 2 follicular waves, and the remaining 32 % exhibited 3 waves during an interovulatory interval (IOI) [49]. Only the final wave was ovulatory, while all preceding waves were anovulatory. Follicular waves were characterized as major or minor waves [50]. Major waves were those in which one follicle was selected to become dominant over other follicles of the wave, while minor waves were those in which selection of a dominant follicle was not

detected. In women with 2 follicle waves, minor major (- +) and major major (+ +) patterns of follicle wave dynamics were observed. In women with 3 follicle waves, minor minor major pattern (- - +), minor major major pattern (- + +) and major major major (+ + +) patterns were observed [50]. It is not known whether ultrasonographically-detectable changes in the endometrium differ between women with 2 versus 3 follicle waves and among women with major and minor patterns of follicular wave dynamics. This information would increase our understanding about the cyclic changes in ovarian and endometrial function that occur in women, and the variability in endometrial development observed among women during the menstrual cycle. We anticipate that this information may also provide insight into potential associations between endometrial growth and probability of conception following controlled ovarian hyperstimulation, ovulation induction, and/or in vitro fertilization for the treatment of infertility.

The objective of this study was to characterize changes in the endometrium every day during one interovulatory interval (IOI) using high-resolution transvaginal ultrasonography. The research hypothesis tested was that endometrial development (as determined by measurements of endometrial area, perimeter, thickness, and echotextural pattern) would differ between women with 2 versus 3 follicular waves and among women with different follicle wave patterns.

6.3 Materials and Methods

Fifty women participated in a study designed to characterize ovarian follicular wave dynamics during the menstrual cycle [49]. Data collected from these 50 women were evaluated to elucidate associations between patterns of follicle wave dynamics and endometrial development Participants were assessed, by history and physical examination, to be healthy women of reproductive age (mean age $\pm$ SD = 28.0 $\pm$ 6.9 years, range = 19 - 43 years). Women who were currently pregnant or had been pregnant or lactating 6 months prior to initiating study procedures, had used hormonal contraception 3 months before enrolling, had a history of irregular menstrual cycles, were taking medication(s) known or suspected to interfere with reproductive function, or were planning surgery during the study period were not eligible to participate.

Informed consent was obtained from all women prior to initiating study procedures. Study protocol was approved by the Institutional Review Board of the University of Saskatchewan.

Each participant underwent daily transvaginal ultrasonographic evaluation of her ovarian and uterine status for one IOI. Scans were initiated 12 days after menses (i.e., before the first ovulation) and were continued until 3 days after the second ovulation. High-resolution Ultramark 9 and ATL HDI 5000 ultrasound machines with 5-9 MHz multi-frequency convex array transducers were used to acquire imaging data. Approximately 90% of the examinations were performed by a single sonographer (ARB). A second sonographer (RAP) was available when the primary sonographer was not present.

The area, perimeter and thickness of the endometrium were measured during each ultrasound examination. Endometrial area and perimeter measurements were based on the shape of an ellipse, in an approximately transverse plane. Endometrial thickness was measured as the distance from anterior stratum basalis-myometrial junction to the posterior stratum basalis-myometrial junction, in mid-sagittal plane. The transverse and sagittal planes of section which represented the largest dimensions of the fundal aspect of the endometrium were used for all measurements. Endometrial echotexture was assessed each day as either an M, A, B, C, or D pattern. The criteria used to determine endometrial pattern are shown in Table 6.1 [20].

Table 6.1: Characteristics for determining endometrial pattern

Pattern	Criteria
M	Active menstrual flow observed
A	Post-menstrual; thin; single line; no detectable differentiation of stratum functionalis and basalis
B	Early follicular phase; triple-line; some differentiation of the stratum funcionalis and basalis
C	Peri-ovulatory; thick; pronounced triple-line; pronounced differentiation of the stratum functionalis and basalis
D	Luteal phase; thick; homogeneous echogenicity; secretory glands observed

Mean profiles were created for each endometrial endpoint during the IOI, irrespective of follicle wave status. Endometrial data were then categorized as belonging to women with 2- or 3- wave cycles. In women with 2 follicle waves, endometrial data were further categorized into - + and + + follicle wave patterns. In women with 3 follicle waves, data were further categorized into - - +, - + + and +++ follicle wave patterns. Repeated measures analyses (PROC MIXED, SAS/STAT Software, 2001) were used to assess changes in the area, perimeter, thickness and pattern of the endometrium during the IOI to determine if differences could be detected between women with 2 versus 3 follicular waves and among women with different follicle wave patterns.

6.4 Results

The overall area, perimeter, thickness and pattern of the endometrium (irrespective of follicle wave dynamics) remained constant during the early to mid luteal phase, decreased approximately 10 days after ovulation (i.e., the late luteal phase), and then increased with the onset of menses during the follicular phase. Endometrial area reached peak values of 281.7 $\pm$ 11.9 mm^2 on the day of the first ovulation, declined to a nadir of 106.8 mm^2 3 days after menses began and reached a peak level again of 253.0 $\pm$ 14.2 mm^2 immediately prior to the second ovulation. Endometrial perimeter reached a peak level of 75.9 $\pm$ 2 mm 10 days after ovulation, decreased to 55.3 $\pm$ 1.8 mm 1 day after menses began and then increased to 66.6 $\pm$ 2.1 mm prior to the second ovulation. Endometrial thickness reached a peak of 10.4 $\pm$ 0.3 mm on the day of the first ovulation, decreased to 4.4 $\pm$ 0.2 mm 1 day after menses began and then increased to 9.2 $\pm$ 0.4 mm in the late follicular phase before the second ovulation. The endometrium was a D pattern 1 day following the first ovulation, decreased to an A pattern 2 days after menses began and became a C pattern in the late follicular phase prior to the second ovulation. Ultrasonographic characterizations of endometrial pattern in one woman during the IOI are shown in Figure 6.1 (A-E).

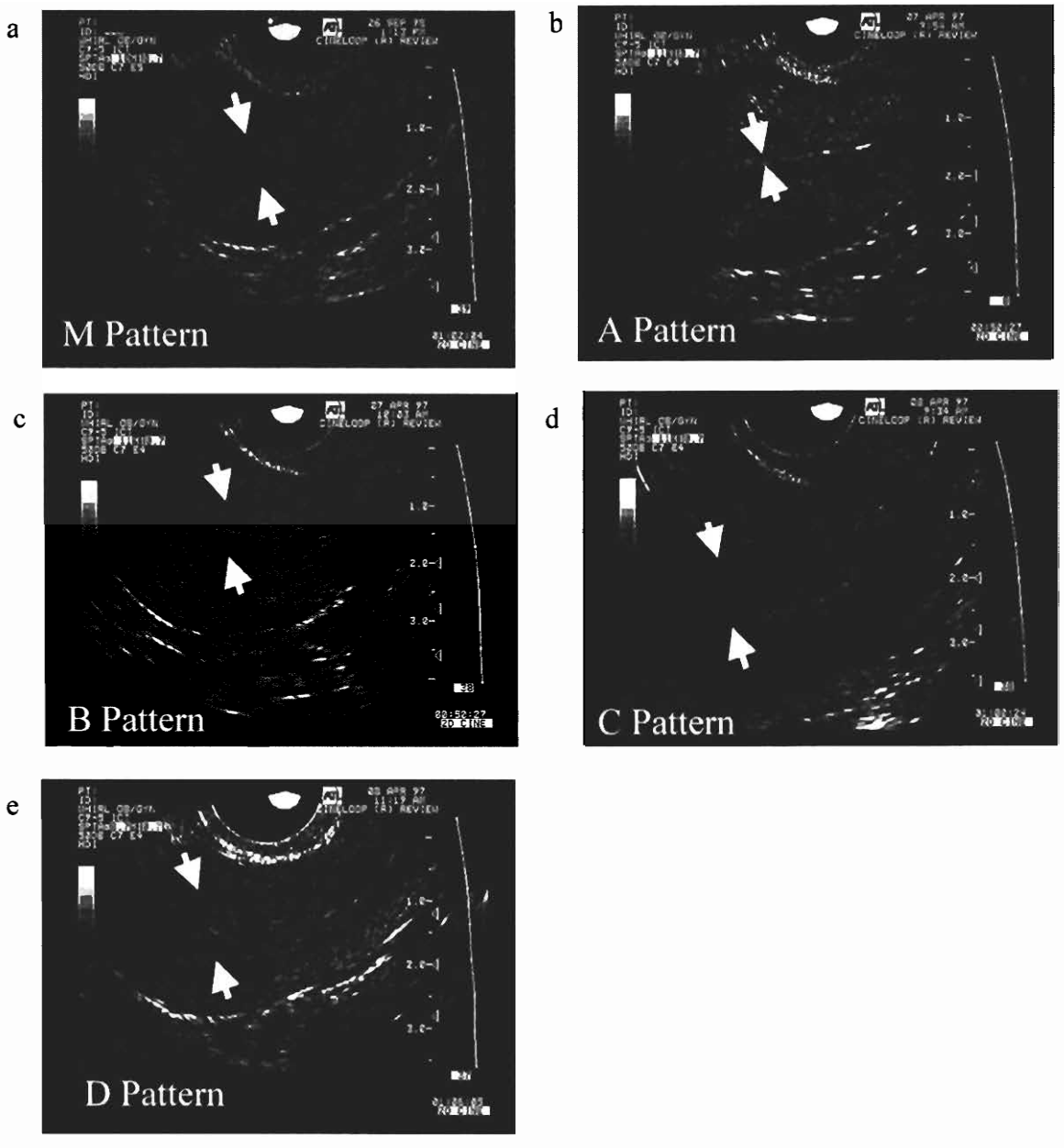

Figure 6.1: Ultrasonographic images of the endometrium illustrating the M pattern (a; day 3 of menses; active flow visualized), A pattern (b; early-follicular phase), B pattern (c; mid-follicular phase), C pattern (d; peri-ovulatory period) and D pattern (e; mid-luteal phase) of echogenicity. Arrows demarcate the anterior and posterior borders of the endometrium.

Changes in endometrial endpoints during the IOI for women with 2 versus 3 follicular waves are illustrated in Figure 6.2 (A-D). Endometrial area (Figure 6.2 A) during the follicular phase of the cycle (i.e., days 17-30) was greater in women with 2 versus 3 follicular waves (day effect: $p<0.0001$, wave effect: 0.88, day*wave effect=0.008). Endometrial perimeter (Figure 6.2 B) during the follicular phase was greater in women with 2 versus 3 follicle waves (day effect: $p<0.0001$, wave effect: $p=0.28$, day*wave effect: $p=0.008$). Endometrial thickness (Figure 6.2 C) during the follicular phase was greater in women with 2 versus 3 follicle waves (day effect: $P<0.0001$, wave effect: $p=0.01$, day*wave effect: $p=0.08$). Likewise, endometrial pattern (Figure 6.2 D) during the follicular phase was greater in women with 2 versus 3 follicular waves (day effect: $p<0.0001$, wave effect: $p=0.02$, day*wave effect: $p<0.0001$). No differences in endometrial endpoints between women with 2 versus 3 waves were detected during the luteal phase ($p>0.05$).

Changes in endometrial endpoints during the IOI for women with major and minor wave patterns of follicle development are show in Figure 6.3 (A-D). In women with 2 follicle waves, endometrial area (day effect: $p<0.0001$, pattern effect: $p=0.15$, day*pattern effect: $p=0.002$) and perimeter (day effect: $p<0.0001$, pattern effect: $p=0.003$, day*pattern effect: $p=0.69$) during the follicular phase were greater in those with + + versus - + wave patterns of follicle growth. In women with 3 follicle waves, no differences in endometrial endpoints were detected among - - +, - + + and + + + follicle wave patterns (day effect: $p<0.0001$, pattern effect: $p>0.05$, day*pattern effect: $p>0.05$).

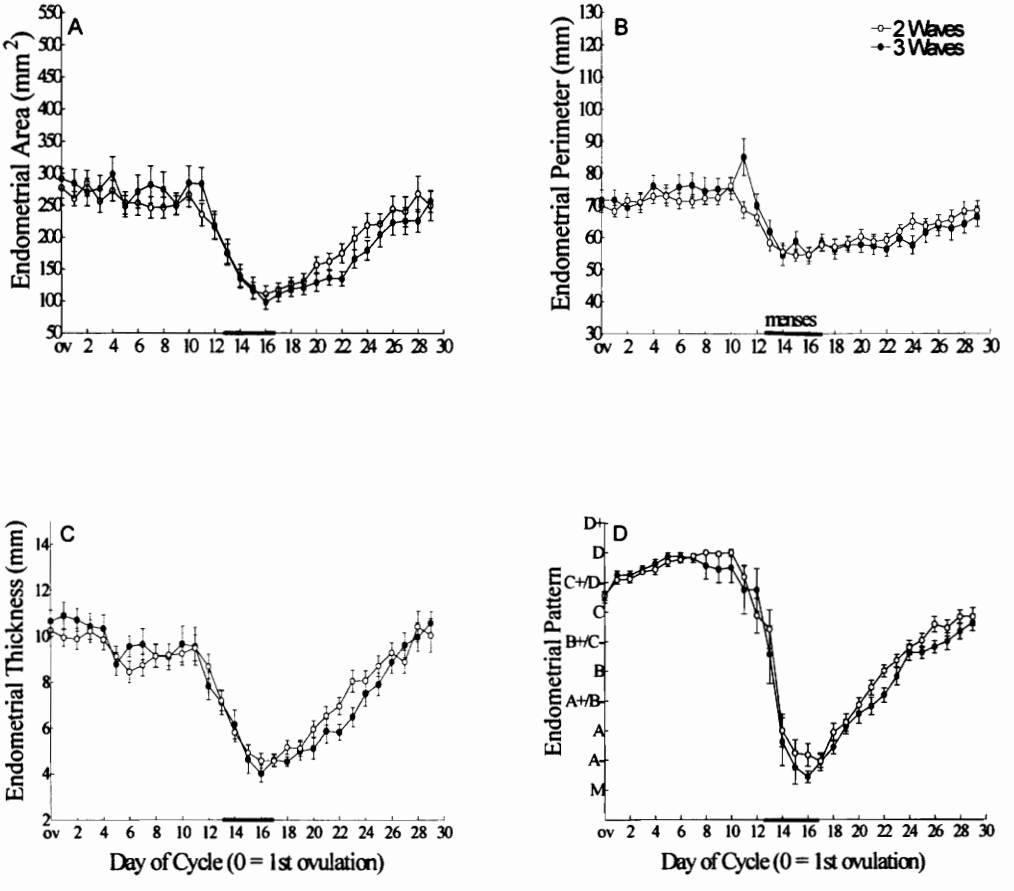

Figure 6.2: Endometrial area (A), perimeter (B), thickness (C) and pattern (D) throughout the interovulatory interval for women with 2 (o) and 3 (•) waves of follicle development.

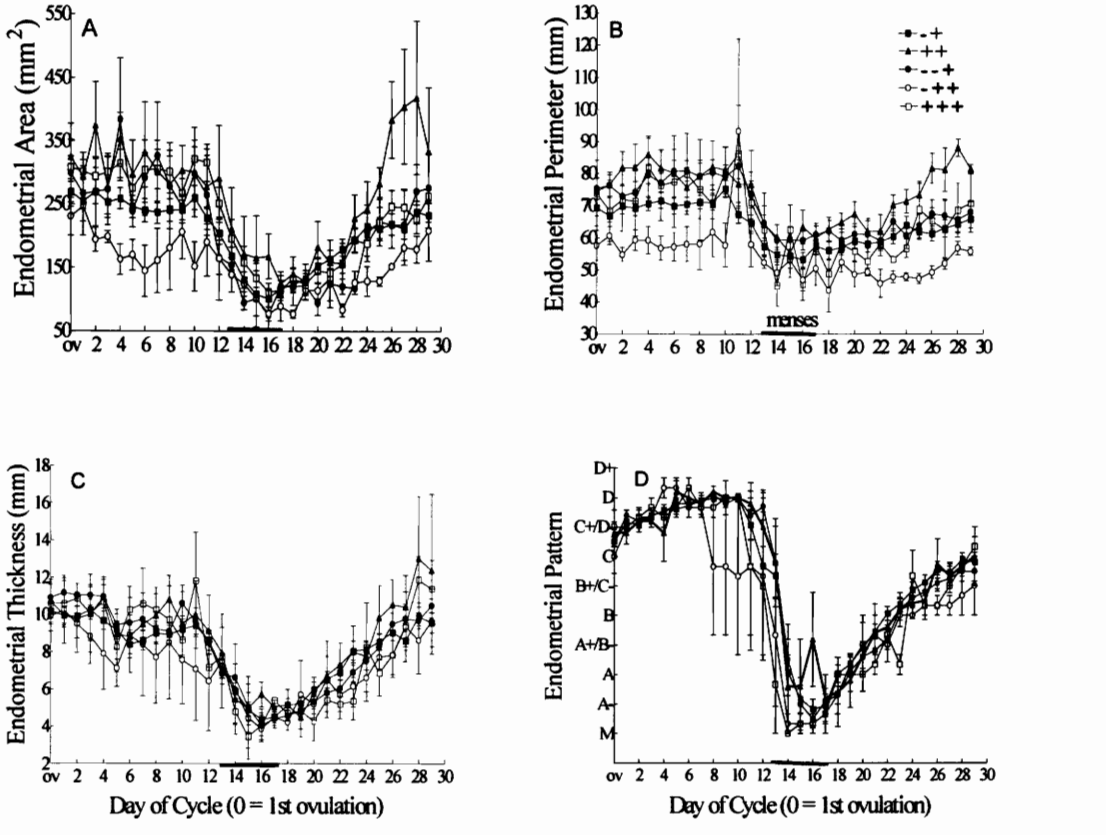

Figure 6.3 Endometrial area (A), perimeter (B), thickness (C) and pattern (D) throughout the interovulatory interval for women with - + (■), + + (▲) - - + (•), - + + (o) and + + + (□) patterns of follicle wave dynamics.

6.5 Discussion

Serial examinations of the endometrium using high-resolution transvaginal ultrasonography supported the results of previous studies which characterized changes in endometrial thickness and echotexture during the menstrual cycle [14, 18, 20, 22-24]. Endometrial area, perimeter and thickness reached a plateau after ovulation, declined at the end of the luteal phase before menstruation, and then increased sharply during the follicular phase of the IOI. Endometrial echotexure was represented by a D pattern in the luteal phase, M pattern during menses, A pattern in the early follicular phase, and C pattern in the late follicular phase of the IOI.

The results of the present study supported the hypothesis that endometrial development would differ among women in association with differences in ovarian follicular wave dynamics. Ultrasonographically-detectable changes in the endometrium during the menstrual cycle were observed in women with 2 versus 3 waves of ovarian follicular development. Endometrial area, perimeter, thickness and pattern measurements increased earlier during the follicular phase in women with 2 waves of follicular development. Earlier development of the endometrium during the follicular phase in women with 2 follicle waves occurred in association with an earlier rise in serum estradiol levels, as previously described [50]. The earlier increase in estradiol levels was attributed to the earlier emergence of the dominant ovulatory follicle in women with 2 versus 3 follicle waves [50]. The pre-ovulatory estradiol, FSH and LH surges occurred 1 day earlier, in association with a shorter IOI, in women with 2 versus 3 follicle waves [50]. We therefore concluded that the earlier emergence of the dominant ovulatory follicle in women with 2 versus 3 follicle waves was associated with an earlier rise in dominant follicle estradiol production, greater endometrial development and an earlier pre-ovulatory hormonal surge.

Changes in endometrial growth, as determined ultrasonographically, were detected in women with minor and major wave patterns of follicle development. In women with 2 follicle waves, endometrial area and perimeter during the follicular phase were greater in women with + + versus - + wave patterns of follicular growth. However, in women with 3 wave patterns of follicle growth (i.e., - - +, - + + and + + + patterns), no differences in endometrial endpoints were observed. Major waves were

those in which a dominant follicle was selected for preferential growth, while minor waves were those in which dominance was not manifest. Follicular dominance is associated with increased estradiol production [48, 51, 52]. Therefore, it seemed likely that greater estradiol levels in women with a + + versus - + pattern of follicle growth were responsible for greater development of the endometrial lining. We expected the endometrium to develop to a greater extent in women with + + versus - + wave patterns during the luteal phase because that is where differences in follicle development occurred (i.e., the development of a major versus minor anovulatory follicle wave prior to the ovulatory wave). We, however, observed greater endometrial growth during the follicular phase. Reasons for this phenomenon are unknown and require further investigation.

The results of the present study have increased our understanding of the basic physiologic mechanisms underlying ovarian and uterine function during the menstrual cycle, and provided rationale for the notion that endometrial development may be related to ovarian follicle wave dynamics. The knowledge that the endometrium develops to a greater extent in women with 2 versus 3 follicle waves may help to explain the variability in endometrial thickness and echotexture that has been reported in women undergoing assisted reproductive technologies (ART), and the inconsistent findings for the use of endometrial growth as a predictor for the probability of conception following ART. In addition, we believe that the knowledge about variability in endometrial growth and follicle wave dynamics during the menstrual cycle will provide insight into uterine factors which may be associated with infertility and/or recurrent pregnancy loss.

6.6 Acknowledgments

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Chapter 7

OVARIAN FOLLICULAR DEVELOPMENT DURING THE USE OF ORAL CONTRACEPTION

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7.1 Abstract

Objective: To evaluate ovarian follicular development in women using oral contraception (OC). The research hypothesis was that women would develop follicles to an ostensibly ovulatory diameter and ovulate during compliant OC use. We further hypothesized that the incidence of follicle development would be greatest during the hormone-free interval (HFI) and in women taking low EE dose OC formulations (20 μg EE).

Materials and Methods: Thirty-six healthy women of reproductive age were randomized to receive either: 1. [35 μg Ethinyl Estradiol (EE,21)/180 μg Norgestimate(NGM,7), 215 μg NGM(7), 250 μg NGM(7)], 2. [30 μg EE(21)/150 μg Desogestrel (DSG,21)] or 3. [20 μg EE(21)/100 μg Levonorgestrel (LNG,21)]. Transvaginal ultrasonography was performed every third day for 3 consecutive 28-day cycles to monitor follicular development. If a follicle reached ≥ 14 mm, ultrasonography was performed daily and blood drawn every other day to determine estradiol-17ß concentrations.

Results: Seventeen of 36 women (47%) grew follicles ≥ 10 mm (i.e., dominant follicles). Nine of the 17 women (53%) grew follicles ≥ 14 mm in association with increased concentratsions of estradiol. Thirty-seven out of 43 (86%) dominant follicles detected emerged during the 7-day hormone free interval (HFI). Maximum follicle diameter and number of dominant follicles observed were greater in women taking lower dose EE OC regimens (20 μg EE) compared to women taking higher dose regimens (30-35 μg EE). No ovulations were observed.

Conclusions: Our results supported the hypothesis that follicular development to an ostensibly ovulatory diameter would occur during OC use. However, no ovulations were detected. Ovarian follicular development during OC use was associated with loss of endocrine suppression during the HFI, rather than user non-compliance.

7.2 Introduction

Oral contraception (OC) is one of the most commonly used and widely studied methods of family planning. Combined oral contraceptives contain orally-active estrogen and progestin which provide negative feedback on the hypothalamo-pituitary axis to prevent ovulation and subsequent pregnancy. It is generally accepted that both steroid components of combined OC act synergistically to suppress the hypothalamo-pituitary axis [1]. It is believed, although not fully elucidated, that the estrogen component of OC inhibits FSH activity and suppresses ovarian follicular development [2, 3], while the progestin component inhibits the LH surge and ovulation [4-6]. It is further believed that OC elicit secondary inhibitory effects on endometrial development, cervical mucous viscosity, cervical dilation, and oviductal motility [7]. Most combined OC regimens consist of 21 days of dosing followed by a 7-day hormone-free interval (HFI) in which menstruation occurs before beginning the next cycle of OC use.

Oral contraception has been reported to be more than 99% effective during perfect use [8] and approximately 90% effective during "real-life use [9]. The primary cause of 'pill failures' (i.e., pregnancy during OC use) has been believed to be user non-compliance [9]. Specifically, women who miss doses, take medications that interfere with OC metabolism, or switch from one OC formulation to another incorrectly are believed to be at greater risk of developing follicles which may ovulate and result in pregnancy [7].

Growth of ovarian follicles to diameters $\geq$ 10 mm has been documented in 23-90% of combined OC users [10: review]. The variability in the incidence of follicle growth has been attributed to differences in the techniques used to detect follicle growth. In early studies, urine and serum endocrine levels were used to indirectly evaluate follicle growth and ovulation in women taking OC. Transabdominal ultrasonography was also used intermittently to determine follicle activity during OC use. In more recent studies, however, high-resolution transvaginal ultrasonography has been used to more accurately visualize follicular development during OC use. Studies to serially monitor follicular development and ovulation during OC use are limited. The development of ovarian follicles to diameters $\geq$ 10 mm is of physiological significance because selection of a dominant follicle for preferential growth and ovulation has been

reported to occur at a diameter of approximately 10 mm during natural menstrual cycles [11]. Selected follicles have been shown to exhibit 'functional dominance' as they secrete estradiol to stimulate their continued development and suppress the growth of the other subordinate follicles during spontaneous menstrual cycles [12, 13]. Studies designed to evaluate follicular development during OC use have revealed that some dominant follicles failed to reach pre-ovulatory diameters (16-22 mm), while others ovulated, reached pre-ovulatory diameters but failed to ovulate, or developed into large anovulatory follicular cysts or 'follicle-like structures' [14-32].

Follicle development has been observed during the HFI of OC regimens. Follicle growth during the HFI has been shown to resemble that observed during the early follicular phase of the natural menstrual cycle [32-34]. In the absence of exogenous steroids, FSH levels rose above the threshold for ovarian stimulation allowing gonadotropin dependent follicle growth and estradiol production [33, 34]. If a dominant follicle did not develop during the HFI, FSH levels fell below threshold when OC were recommenced and follicular development was further suppressed. If a dominant follicle developed during the HFI, it continued to develop despite lowered FSH levels after OC use was reinitiated. Ovulation of follicles that developed during the HFI was believed to be prevented by inhibition of the LH surge [34]. In contrast, others have reported ovulation of follicles that developed during the HFI [35].

Follicular development, ovulation and anovulatory follicular cysts have been well documented following OC dosing errors [36-43]. The time during the cycle when dosing errors occur influences OC efficacy. Missing the last few dosing pills of a cycle and/or the first dosing pills of consecutive cycles extends the HFI, and has been associated with an increased incidence of dominant ovarian follicles and elevated serum estradiol-17β levels [36-38, 42, 43]. In contrast, shortening the HFI has been associated with decreased follicular development and lower estradiol-17β levels [35, 44].

Contraceptive efficacy may also depend on the dose of steroid hormones comprising OC formulations. The first OC approved by the FDA in 1960 (Enovid) contained 150 μg Mestranol and 9.85 μg norethynodrel [45]. The use of Enovid was later associated with unwanted cardiovascular risks [46, 47]. As a result, Ethinyl Estradiol (EE) was incorporated into OC rather than Mestranol, and the estrogen dose

was reduced to 50 μg and eventually 30-35 μg. Recently, formulations containing EE doses as low as 20 μg have been marketed to further reduce the incidence of unwanted side effects. In addition, the development of second, third and fourth generation progestins has resulted in better-tolerated OC regimens [7]. There is increasing evidence to suggest that the degree of pituitary-ovarian suppression is related to the dose of estrogen, while the type and dose of progestin are less important [28, 33]. Pituitary-ovarian suppression has been reported to be compromised when EE doses as low as 20 μg are used [28, 48, 49]. The increased risk of follicle development and ovulation after missed OC doses is believed to be greater in women taking formulations containing $\leq$ 20 μg EE [32]. Studies to ultrasonographically and endocrinologically evaluate the effect of different progestins on ovarian function, while properly controlling for the estrogen component, however, are lacking and emphasize the need for continued research.

Ovarian follicular development and ovulation during OC use are not fully understood. Follicle development during OC use has been attributed to user non-compliance, most notably in women taking low EE dose regimens (i.e., $\leq$ 20 μg EE) [32, 39, 40 , 41, 50-52]. We recently documented pre-ovulatory follicular development and ovulations in women during proper and improper dosing of combined OC [53]. These observations led us to believe that the development and ovulation of dominant follicles was a common phenomenon during compliant OC use.

The objective of the present study was to characterize ovarian follicular development during proper dosing of 3 different OC formulations using high-resolution transvaginal ultrasonography. We hypothesized that the development and ovulation of dominant follicles would occur during compliant OC use, regardless of the regimen used. We hypothesized that follicular development would be detected most often during the HFI. We hypothesized that women taking lower EE dose OC (20 μg EE) would exhibit greater follicular development than women taking moderate EE dose OC (30-35 μg EE). In addition, we hypothesized that a greater incidence of follicular activity in the low EE dose treatment group compared to the moderate EE dose groups would occur in association with greater endometrial development.

7.3 Materials and Methods

This study was a single center, randomized open-label trial. Thirty-six women between the ages of 18 and 35 years (mean age $\pm$ SD = 24.5 $\pm$ 0.8 years) with a history of regular menstrual cycles were enrolled in the study. Participants were assessed, by history and physical examination, to be healthy women with no contraindications to OC use. Women who were using OC at the time of screening or had used OC within the previous 3 months were not eligible to participate. Informed consent was obtained from all women prior to initiating study procedures. The study protocol was approved by the Institutional Review Board at the University of Saskatchewan.

Each woman was randomized to receive one of the following three OC formulations: 1. [35 μg Ethinyl Estradiol (EE, 21)/180 μg Norgestimate (NGM, 7), 215 μg NGM (7), 250 μg NGM (7)], 2. [30 μg EE (21)/150 μg Desogestrel (DSG, 21)] or 3. [20 μg EE (21)/100 μg Levonorgestrel (LNG, 21)]. Oral contraception was initiated on the first day of menses, and continued for 3 consecutive 28-day cycles. Each cycle consisted of 21 days of dosing pills followed by a hormone-free interval of 7 days. Twelve women were randomized into each treatment group. Volunteers were assigned a diary card each cycle to record OC initiation, missed pills and adverse events. Diary cards were reviewed frequently with the research volunteers throughout the course of the study period.

Ovarian follicular development was monitored every third day during the 3-month study period using high-resolution transvaginal ultrasonography. At each visit, the diameters of all follicles $\geq$ 2 mm were recorded. The thickness and echotextural pattern (i.e., M, A, B, C or D) of the endometrium were also recorded [54]. If a follicle grew to $\geq$ 14 mm, daily ultrasound examinations were performed until the follicle either ovulated, regressed or remained the same size for 3 days. Blood was drawn every other day from the time the follicle reached 14 mm until it completely regressed, to determine circulating concentrations of estradiol-17β. Ovulation was defined as the disappearance of a large follicle (>15 mm) that had been identified by ultrasonography on the previous day and the subsequent visualization of a corpus luteum [55].

The growth and regression profiles of all follicles that grew $\geq$ 10 mm (i.e., dominant follicles) were graphed for each treatment group. Follicle emergence was

defined as the day on which each follicle was first identified at a diameter of 4-5mm. Maximum follicle diameter and the numbers of follicles ≥ 10 mm and ≥ 14 mm in each of the treatment groups were statistically compared using Non-Parametric analyses (i.e., Kruskall-Wallis, Mann-Whitney and Chi-Square Tests; SPSS Version 11, 2002). Endometrial thickness and pattern during the study period were compared among the treatment groups using Repeated Measures analyses of variance (PROC MIXED, SAS/STAT, 2001).

7.4 Results

Seventeen of the 36 (47%) women evaluated grew follicles ≥ 10 mm (i.e., dominant follicles) during the 3 month study period. Four of the 43 dominant follicles (9.3 %) were present at the time OC use was initiated. More dominant follicles emerged during cycles 1 (18/43 follicles; 41.9%) and 2 (15/43 follicles; 34.9%) compared to cycle 3 (6/43 follicles; 14.0%), (p=0.05). In 9/108 cycles, missed pills were reported. Missed pills were not associated with the growth of dominant follicles.

In 8/17 (47%) women that developed dominant follicles, the follicles regressed before reaching 14 mm. In the remaining 9/17 (53%) women that developed dominant follicles, the follicles grew to diameters ≥ 14 mm and either regressed (n=11/12 follicles) or formed a hemorrhagic anovulatory follicle (n=1/12 follicles). No ovulations were observed. All of the anovulatory dominant follicles observed during the study regressed before the end of cycle 3.

The growth profiles of all dominant follicles that developed in each of the 3 treatment groups are illustrated in Figure 7.1 (A-C). We found that 37/43 (86%) of dominant follicles emerged during the 7-day hormone free interval (HFI). Specifically, 6/7 follicles in the EE/NGM group (85.7%), 12/13 follicles in the EE/DSG group (98.9%) and 19/23 follicles in the EE/LNG group (82.6%) emerged during the HFI.

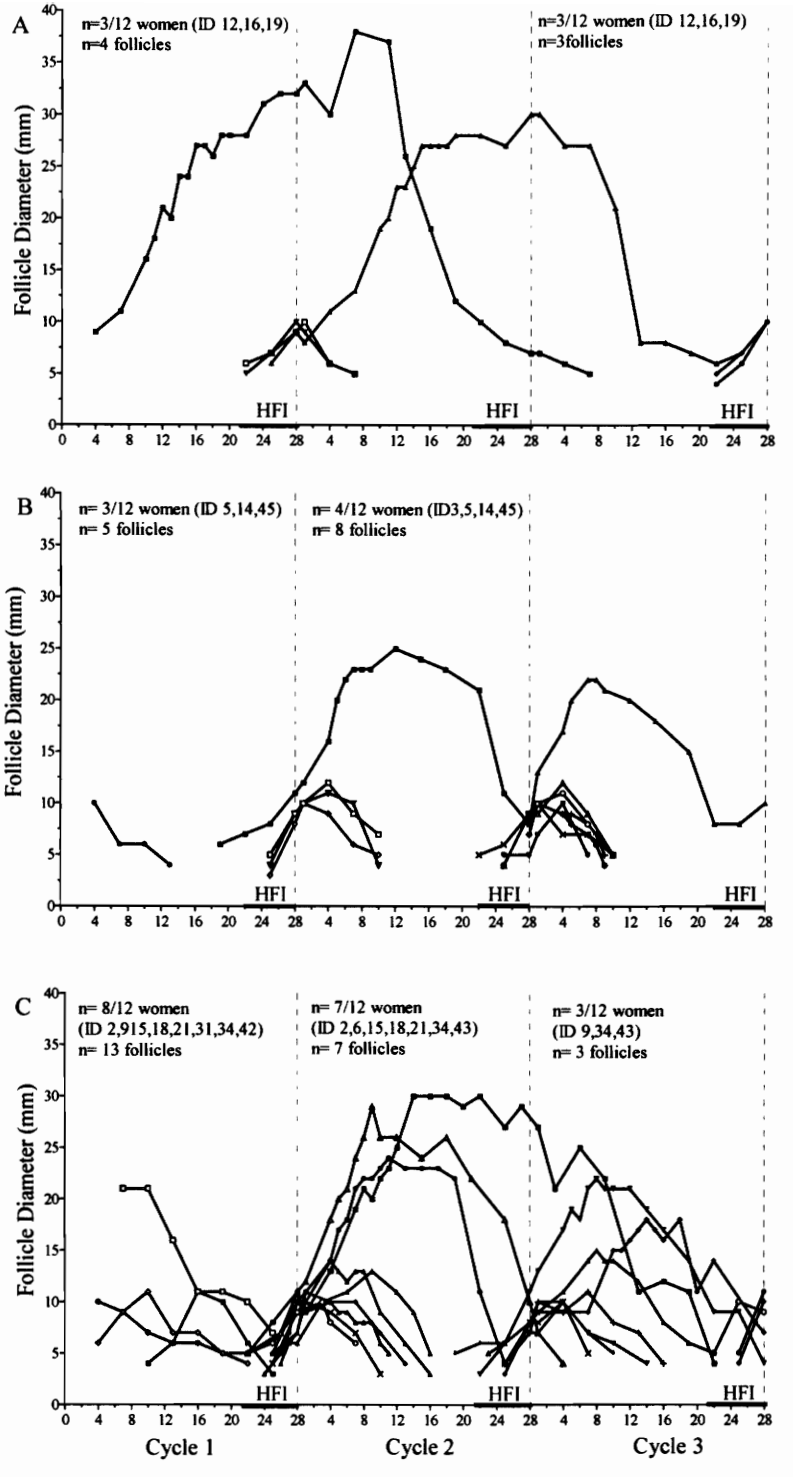

Figure 7.1: Growth profiles of all follicles that grew ≥ 10 mm during the study period for women randomized to the EE/NGM (A; n=12), EE/DSG (B; n=12) and EE/LNG (C; n=12) treatment groups. The number of women that developed dominant follicles and the number of dominant follicles that were observed during each cycle are indicated on the graph. HFI = hormone-free interval.

Maximum follicle diameter and the number of follicles ≥ 10 mm and ≥ 14 mm in each of the treatment groups are shown in Table 7.1. Maximum follicle diameter was greater in women randomized to the EE/LNG group compared to the EE/NGM and EE/DSG groups ($p<0.05$). The EE/LNG formulation was associated with the development of more follicles to diameters ≥ 10 mm and ≥ 14 mm, compared to the EE/NGM and EE/DSG groups ($p<0.05$). Women randomized to the EE/DSG group grew more follicles ≥ 10 mm than women in the EE/NGM group ($p<0.05$).

Table 7.1: Maximum follicle diameter (mm) and the number of follicles which grew to ≥ 10 mm or ≥ 14 mm, compared among the 3 OC formulations.

	EE/NGM	EE/DSG	EE/LNG
Maximum follicle diameter (mm)	12.1±1.4 [a] (mean rank=14.3)	10.3±1.4 [a] (mean rank=15.9)	16.4±2.3[b] (mean rank=25.4)
Number of follicles ≥ 10 mm	7 [a]	13 [b]	23 [c]
Number of follicles ≥ 14 mm	2 [a]	2 [a]	8 [b]

[a,b,c] Within rows, values with different superscripts are significantly different ($p<0.05$).

The growth profiles of all individual follicles that grew to diameters > 14 mm and the corresponding concentrations of estradiol-17β are shown in Figure 7.2 (A-I). Estradiol-17β increased to a mean maximum concentration of 630.6 ± 112.5 pmol/mL (range = 130 - 1350 pmol/mL), in association with the growing phase of follicle development. Follicles grew to a diameter at which they lost functional dominance (i.e., estradiol levels abruptly fell), after which time they either regressed immediately or continued to grow for a few days before regressing.

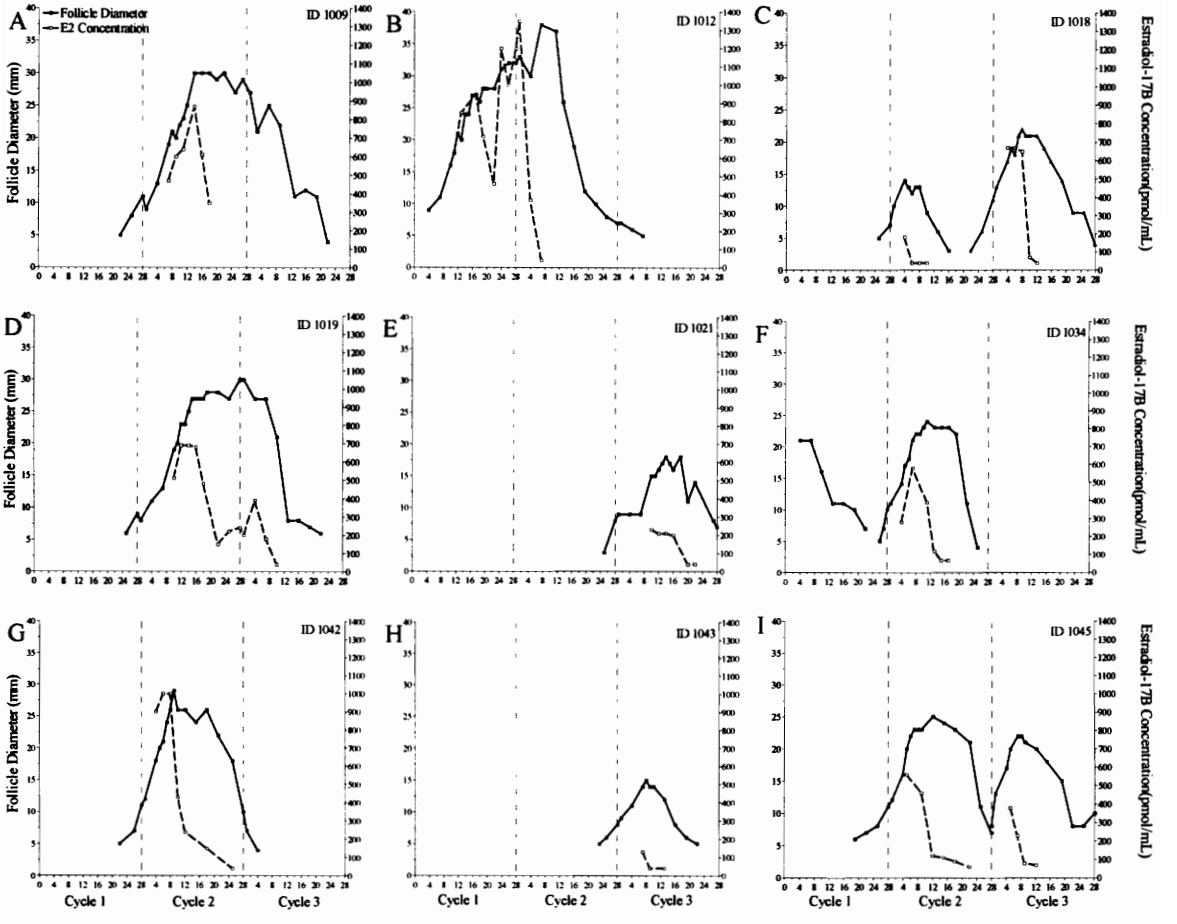

Figure 7.2: Growth profiles of all follicles that grew to diameters of ≥ 14 mm during the study (solid lines), and the corresponding serum estradiol-17β concentrations (n=9 women; A-I; dashed lines).

Endometrial thickness and pattern profiles throughout the study for the 3 treatment groups are illustrated in Figure 7.3 (A,B). Endometrial thickness and pattern increased during the HFI and then regressed during each cycle of OC dosing. However, changes in endometrial thickness and pattern during the study period were not different among the 3 treatment groups (day effect: $p<0.0001$, treatment by day interaction effect: $p>0.05$).

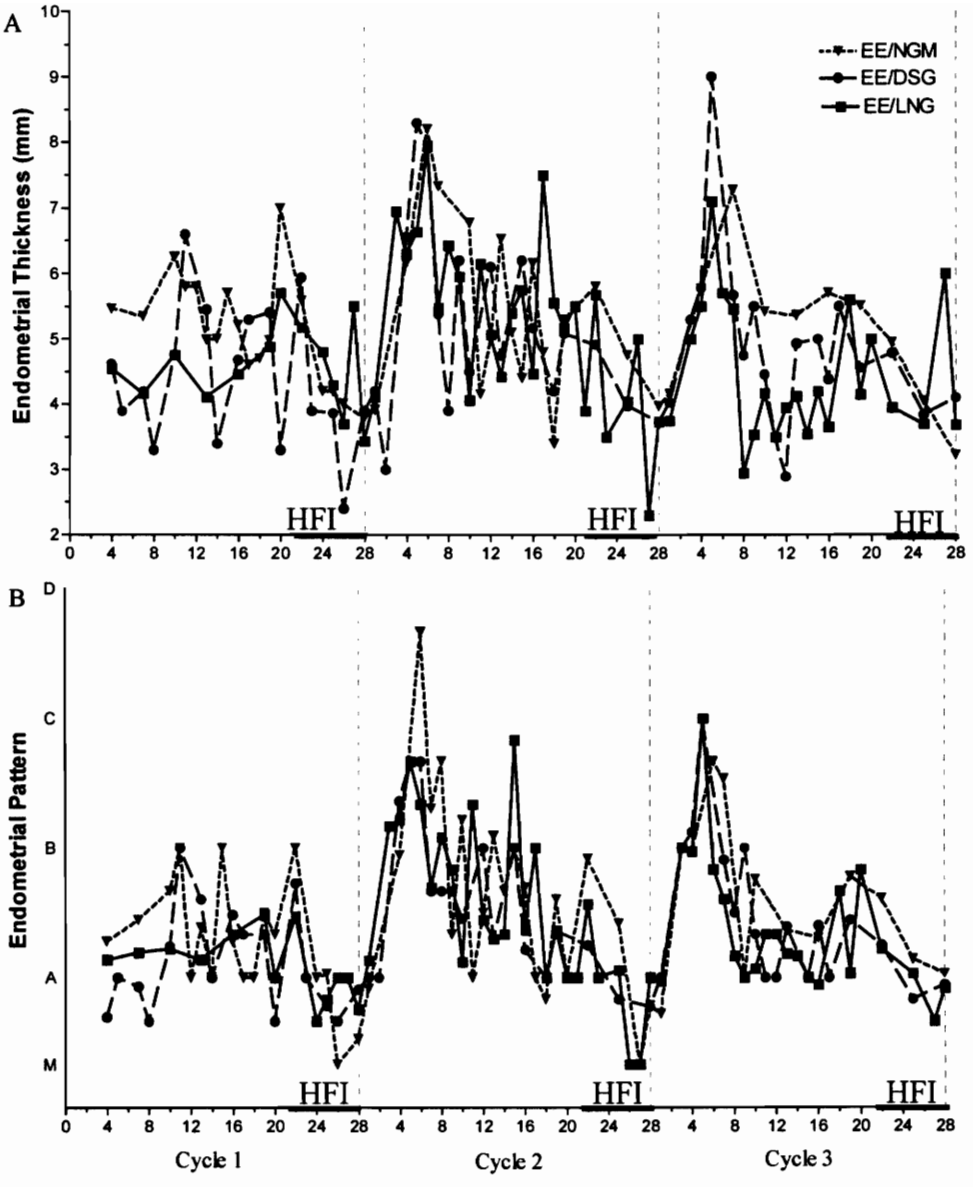

Figure 7.3: Endometrial thickness (A) and pattern (B) during the study period for women randomized to the EE/NGM (▼; n=12), EE/DSG (●; n=12) and EE/LNG (◆; n=12) treatment groups. HFI = hormone free interval.

7.5 Discussion

Ovarian follicular development to an ostensibly ovulatory diameter occurred during the compliant use of OC. Our hypothesis that ovulation would occur during compliant OC use was not supported in the present study. All of the follicles that reached pre-ovulatory diameters failed to ovulate. Rather, they regressed or formed hemorrhagic anovulatory follicles.

The growth profiles and serum estradiol-17β profiles of follicles ≥ 14 mm during the growth phase to 16-22 mm markedly resembled those of ovulatory follicles during natural menstrual cycles previously observed in our laboratory [11]. Similarly, dominant follicles observed in the present study appeared ultrasonographically indistinguishable from those observed during spontaneous menstrual cycles [11]. Follicle growth to an ostensibly ovulatory diameter was accompanied by an increase in dominant follicle estradiol production. We therefore believe that these follicles had the potential to ovulate. Contraceptive efficacy may have been maintained by suppressing the LH surge and subsequent ovulation, as speculated in other reports [32, 34]. Although no ovulations were observed in the present study, ovulations have been observed during compliant OC use [53]. It is not currently known why some follicles ovulate and others do not during OC use. Additional studies are required to determine whether endogenous LH secretion differs between women that develop ovulatory versus anovulatory follicles during OC use. Information about the association between anovulation and LH levels would increase our understanding of the mechanisms underlying OC use and also provide insight into anovulatory infertility.

Follicle development during OC use was associated with loss of endocrine suppression during the HFI, rather than user non-compliance as previously speculated [9]. Eighty-six percent of dominant follicles that were observed during the study emerged during the HFI, regardless of the OC regimen used. These findings corroborate with previous reports which suggest that follicles can easily develop to pre-ovulatory diameters when exposed to an FSH rebound [34]. Small antral follicles (4-5 mm) appear to be capable of responding to FSH levels above the threshold for ovarian stimulation, even after prolonged periods of suppression with exogenous estrogen and progesterone. The frequent emergence of dominant follicles in the HFI advocates for a

reduction in the duration of the HFI or the use of continuous OC regimens in which the HFI is completely eliminated.

The lower dose 20 μg EE formulation was associated with a lesser suppressive effect on follicular growth than the 30 and 35 μg formulations. Women administered the 20 μg EE regimen grew more follicles ≥ 10 mm and ≥ 14 mm (in association with pre-ovulatory levels of estradiol-17β) than women taking the 30 and 35 μg formulations. In addition, maximum follicle diameter was greater in women administered the 20 μg EE regimen compared to the 30 and 35 μg regimens. Some researchers have indicated that the degree of pituitary-ovarian suppression is related to the dose of estrogen, while the type and dose of progestin appear less important [28, 33]. The results of the present study support the notion that follicle activity during OC use is related to the EE dose. However, in retrospect, it would have been useful to have used 3 different OC containing the same progestin to rule out confounding effects of the progestin component.

Endometrial thickness and pattern increased during the HFI and the first week after the HFI, to levels which compared to those previously observed during natural menstrual cycles (Baerwald and Pierson, unpublished data). Endometrial thickness and pattern declined during the next 2-3 weeks of OC dosing, until the following HFI. The finding that the endometrium was not suppressed during the HFI further supports a reduction or elimination of the HFI in OC regimens. Our hypothesis that endometrial development would be greater in women administered the lower 20 μg EE formulation was not supported. There were no differences in endometrial thickness and pattern profiles among the 3 treatment groups. We interpreted these findings to mean that OC act first at the level of the hypothalamus and pituitary to inhibit gonadotropin secretion, secondarily at the ovary to suppress follicle development, and thirdly at the level of the uterus to suppress growth of the endometrium.

A variable incidence of follicle growth and ovulation during OC use has previously been reported, based on studies in which ovarian activity was measured by urine or serum estrogen and progesterone levels alone or in combination with infrequent ultrasonographic monitoring of follicle growth status. In the present study, we serially monitored the fate of individual dominant follicles that develop during OC use using

high-resolution transvaginal ultrasonography. We demonstrated that follicle development to pre-ovulatory diameters occurs during compliant OC use. The incidence of follicle development was related to loss of endocrine suppression during the HFI and the dose of EE comprising the OC regimen. We evaluated the development of dominant follicles ($\geq$ 10 mm) in this study. However, the development of follicles < 10 mm during OC use has not been studied. Follicle growth in women is a highly dynamic process, in which 2 and 3 waves of antral follicles grow and regress during the natural menstrual cycle [11, 56]. Follicle wave emergence was detected at a diameter of 4-5 mm. It is plausible that waves of antral follicle development may occur during OC use. The follicle wave that has initiated growth at the time of the HFI may be able to continue developing in an endocrine-rich milieu and give rise to a dominant ostensibly ovulatory follicle. Future research should be performed to determine whether waves of follicle development occur in women taking OC. An increased understanding of the processes of follicle recruitment, selection of a dominant follicle, acquisition of functional dominance and loss of dominance that occur during OC use would aid in the development of more efficacious hormonal contraceptive regimens.

7.6 Acknowledgments

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Chapter 8

THE EFFECTS OF ORAL CONTRACEPTION ADMINISTERED AT DEFINED STAGES OF OVARIAN FOLLICULAR DEVELOPMENT

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8.1 Abstract

Objective: To elucidate the effects of initiating oral contraceptives (OC) at different stages of follicle development. We hypothesized that follicular atresia would be induced when OC use was initiated at a follicle diameter of 10 mm. We hypothesized that follicles would ovulate when OC use was initiated later during follicle development at diameters of 14 and 18 mm. Furthermore, we hypothesized that follicular development following OC use would occur in association with endometrial development.

Materials and Methods: Forty-five healthy women between the ages of 18 and 35 were randomized to initiate combined OC use when a follicle diameter of either 10, 14 or 18 mm was detected. The diameters of all follicles $\geq$ 2 mm, and the thickness and pattern (M, A, B, C or D) of the endometrium were recorded using transvaginal ultrasonography on day 2 of menses and intermittently thereafter until randomization status was met. Blood was drawn to measure serum concentrations of estradiol-17ß, luteinizing hormone (LH) before and after initiating OC use, and serum progesterone concentrations after initiating OC use. Follicle dynamics, endocrine concentrations and endometrial growth were compared among and within treatment groups.

Results: No ovulations (0/16) were observed when OC use was initiated at follicle diameters of 10 mm, 4/14 ovulations were observed at 14 mm, and 14/15 ovulations were observed at 18 mm. The remaining follicles in each treatment group regressed or developed into anovulatory cysts. Peak LH and estradiol levels were lowest in the 10 mm group, moderate in the 14 mm group and greatest in the 18 mm group. Endometrial development to normal reference levels occurred after OC initiation at follicle diameters of 14 and 18 mm. Endometrial development was suppressed below normal reference levels in association with follicular atresia in women who initiated OC use at a diameter of 10 mm.

Conclusions: Follicular development, endometrial growth and endogenous endocrine levels were not suppressed effectively when OC use was initiated at mid to late stages of follicular development. The finding that dominant follicles regressed when OC was initiated at a diameter of 10 mm, but ovulated when OC was initiated at diameters of 14 and 18 mm (in association with increased concentrations of estradiol and LH) supported

previously held notions that dominant follicles produce estradiol and respond to increasing concentrations of LH during the mid to late follicular phase of the menstrual cycle. In addition, the results of this study provide rationale for discouraging the use of "Sunday-start" OC regimens and reducing or completely eliminating the HFI in combined OC regimens.

8.2 Introduction

Oral contraceptives (OC) are comprised of orally-active estrogen and progestins which elicit negative feedback effects on the hypothalamo-pituitary axis [1]. The mechanisms of action underlying OC are not fully understood. The estrogen component of OC is thought to inhibit pituitary Follicle Stimulating Hormone (FSH) production and suppress ovarian follicular growth [2, 3], while the progestin component has been shown to inhibit the Luteinizing Hormone (LH) surge and ovulation [4-6]. However, ovarian follicular development is not completely suppressed during OC use. Ovulatory and anovulatory follicles have been reported in women taking OC [7-33], and endogenous estradiol levels have occasionally been reported to reach preovulatory levels [13, 14, 31-35]. It is not currently known why some follicles ovulate during OC use and others do not. It has been speculated that follicles which develop during OC use fail to ovulate due to inhibition of the LH surge [36]. However, research to confirm that theory has not yet been documented.

The degree of follicular activity that occurs during OC use depends upon the type and doses of steroid hormones used. There is increasing evidence to suggest that the degree of pituitary-ovarian suppression is related to the dose of estrogen, while the type and dose of progestin are less important [31, 37]. Pituitary-ovarian suppression may be compromised when EE doses as low as 20 μg are used [31, 38, 39]. However, studies are required to ultrasonographically and endocrinologically evaluate the effect of different progestins on ovarian function.

The incidence of follicle growth during OC use may also depend on the administration scheme used. Starting OC on the first day of menses has been shown to suppress follicular growth [16]. In contrast, many women opt to use the "Sunday Start" regimen, in which they take their first OC dose on the first Sunday after menses begins.

This regimen was designed to minimize the possibility of menstrual bleeding on weekends. With this method, OC use may be postponed until 7 days after menses. However, dominant follicles have been observed within the first 7 days of the menstrual cycle [40]. Therefore, women who opt to delay OC use may be at greater risk of developing follicles which are capable of ovulating.

Follicle development has been observed during the hormone free interval (HFI) of OC regimens. We recently documented that 86% of follicles $\geq$ 10 mm that developed during OC use emerged during the HFI [32]. Follicle development and endogenous FSH levels during the HFI have been reported to reach levels comparable to those observed during the early follicular phase [16, 41-44]. Resumption of OC at the end of the HFI resulted in decreased FSH, despite continued growth of dominant follicles [44]. Missing the last OC doses of the cycle or the first doses of the subsequent cycle extends the HFI, and has been associated with a greater degree of follicle growth [17, 35, 45-48]. The risk of developing follicles following an extended HFI is believed to be more pronounced in women taking low EE dose OC ($\leq$ 20 g EE) [36].

A greater understanding of natural cycle folliculogenesis will help to elucidate follicle development during OC use, and allow the development of safer and more efficacious hormonal contraceptive regimens. Major and minor waves of follicular development have been reported to occur at regular intervals during natural menstrual cycles [40]. Major waves were those in which a dominant follicle was physiologically selected for preferential growth over subordinate follicles. Selection of anovulatory and ovulatory dominant follicles was observed during the menstrual cycle [40]. Follicle selection occurred within the first 3 days following wave emergence at a diameter of approximately 10 mm [40]. Minor waves were those in which selection was not manifest. The emergence of major and minor waves was preceded by a rise in circulating concentrations of FSH [40]. Physiologic selection of a dominant follicle, however, has been shown to occur in association with decreasing FSH levels [49, 50]. It is therefore believed, based on studies performed in animal species, that the dominant follicle becomes selected over subordinate follicles because it exhibits greater FSH sensitivity [50]. At the time of selection, the dominant follicle has been reported to secrete estradiol and inhibin which are believed to prevent the growth of subordinate

follicles [51-53]. Estradiol is believed to induce granulosa cell LH receptor formation, allowing the dominant follicle to become more responsive to LH as it continues to develop during the follicular phase [50, 54-57]. The ability of the dominant follicle to respond to the mid-cycle LH surge determines its capacity to ovulate [58]. Estradiol secreted from the dominant follicle also stimulates the development of the endometrial lining during the follicular phase, in preparation for ovulation [59].

It has been postulated that waves of antral follicular development may occur in women taking OC [32]. The follicle wave that has initiated growth at the time of a delayed OC initiation scheme or during the HFI may be able to continue developing in an endocrine-rich milieu and give rise to a dominant ostensibly ovulatory follicle. Continued investigations are required to determine whether ovarian follicular waves occur during OC use.

Evaluating follicle development during the use of OC allows us to test hypotheses about the basic biology underlying follicle dynamics in women, not otherwise possible. The objective of this study was to elucidate the effects of initiating combined OC use at defined stages of dominant follicle development. We hypothesized that atresia would be induced when OC were initiated at a follicle diameter of 10 mm, and that follicles would develop to pre-ovulatory diameters and ovulate when OC were initiated later during follicle development at diameters of 14 and 18 mm. We hypothesized that atresia would occur in association with low circulating concentrations of LH and estradiol, while ovulations would be associated with pre-ovulatory surges in LH and estradiol. We further hypothesized that follicular development after initiation of OC would be associated with growth of the endometrium.

We anticipated that the results of this study would allow us to determine the risk of follicle development and ovulation after initiating OC use during the mid to late follicular phase of the menstrual cycle, and provide insight into the mechanisms of action underlying OC. In addition, we expected that this information would increase our understanding of the biologic mechanisms underlying follicular growth, ovulation and atresia.

8.3 Materials and Methods

A prospective, randomized, controlled, single-center, open-label trial was conducted. The study protocol was approved by the Institutional Review Board at the University of Saskatchewan, and study procedures were conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. All research participants were non-smoking, healthy women between the ages of 18 and 35 (mean age $\pm$ SD = 25.2 $\pm$ 4.8 years) with no contraindications to OC, as confirmed by a medical history and physical examination. Women who were using OC at the time of screening or had used OC within the previous 3 months were not eligible to participate. Informed consent was obtained from all women before study procedures were initiated.

Forty-five women were enrolled in the study. Ovarian follicular development was evaluated using high-resolution transvaginal ultrasonography (HDI 5000, Advanced Technologies Laboratories: Bothell, WA, USA). At each ultrasound examination, the diameters of all follicles $\geq$ 2 mm were recorded. Follicle diameter was determined by averaging the mean of the follicle length and width in the transverse plane by the mean of the follicle length and width in the sagittal plane. Ovulation was defined as the disappearance of a large follicle (> 15 mm) that had been observed the previous day, and the subsequent visualization of a corpus luteum [60]. Endometrial thickness and pattern were also recorded at each visit [61].

Women were randomly assigned to initiate OC use when a follicle diameter of either 10 mm (n=16), 14 mm (n=14) or 18 mm (n=15) was first detected following menses. All women received the same monophasic OC formulation, which contained desogestrel 150 μg plus ethinyl estradiol 30 μg (days 1-21). Ultrasonography was performed on day 2 of the cycle (day 1 = first day of menses) and intermittently thereafter until randomization status was met. Once OC use was initiated, ultrasound examinations were performed daily until the follicle ovulated, regressed or remained static for 3 days (i.e., determination of follicular fate). Examinations were then performed every 2 to 3 days until the last OC dose or until the follicle had completely regressed. Participants were provided with barrier methods of contraception (condoms) throughout the duration of the study.

Blood was drawn to measure serum concentrations of estradiol-17ß and LH when a follicle diameter of 10 mm was detected in women randomized to the 10 mm group, 10 and 14 mm for women in the 14 mm group, and 10, 14 and 18 mm for women in the 18 mm group. After OC initiation, blood was drawn every other day until follicle fate was determined. Blood was also drawn 6 and 9 days after the determination of follicle fate to measure serum progesterone concentrations. Blood samples were collected into a 7 mL clot-activated tube and allowed to coagulate for 15-30 minutes at room temperature before centrifugation for 10 minutes at 700 G. Serum was drawn off and stored at -20°C. Sequential competitive fluorescence immunoassays (Immulite®, Diagnostic Products Corporation, Los Angeles CA, USA) were performed to measure serum hormone levels. Interassay co-efficients of variation were as follows: estradiol (low=6.8%, medium=7.0% and high=8.7%), LH (low=1.7%, medium=3.2% and high=3.2%) and progesterone (low=7.5%, medium=8.4% and high=7.4%). Minimal detectable limits were 15 pg/mL for estradiol, 0.1 mIU/mL for LH and 0.2 ng/mL for progesterone.

The diameters of all individual follicles that grew to $\geq$ 10 mm during the study period were serially tabulated and graphed for each woman. Follicular fate was categorized as: 1) regression after developing to 10-14 mm, 2) regression after developing to > 14 mm 3) anovulatory follicular cyst, 4) hemorrhagic anovulatory follicle 5) ovulation, or 6) concurrent growth of 2 follicles > 10 mm. Anovulatory follicular cysts were characterized as follicles that developed to diameters of $\geq$ 25 mm, failed to ovulate and regressed. Hemorrhagic anovulatory follicles (HAF) were characterized as follicles which failed to ovulate and hemorrhaged into the follicle lumen (as determined by the ultrasonographic visualization of hyperechoic networks in the lumen representing fibrin clot formation).

Mean follicle diameter, estradiol-17ß and LH profiles were created for all women representing each follicular fate category within each treatment group. Follicle diameter and endocrine data were centralized to the day of randomization for graphical purposes. A subset of data obtained from a previous study, in which follicle development, endometrial growth and serum hormone levels during natural menstrual cycles were characterized, was randomly-chosen as reference data (n=12)[40].

Descriptive statistics for follicle growth profiles were compared within treatment groups and among the reference and treatment groups using independent sample T-tests, one-way analyses of variance and Scheffe's post-hoc tests (SPSS Version 11.0, 2002). Estradiol-17ß levels at follicle diameters of 10, 14 and 18 mm, peak estradiol-17ß levels, peak LH levels and progesterone levels on day 6 and 9 were compared within and among groups using paired T-tests, independent sample T-tests, one-way analyses of variance and Scheffe's post-hoc tests (SPSS Version 11.0, 2002). Endometrial thickness and pattern 2, 6, 10, and 14 days after randomization and peak endometrial endpoints were compared in the same manner.

8.4 Results

No ovulations were observed when OC were initiated at a follicle diameter of 10 mm. Twelve out of 16 women (75%) in the 10 mm treatment group grew follicles 10-14 mm that regressed and 4/16 women (25%) grew follicles > 14 mm that regressed.

When OC were initiated at 14 mm, 5/14 women (36%) ovulated, 3/14 (21%) developed anovulatory follicular cysts, 2/14 (14%) grew follicles 10-14 mm that regressed and 4/14 (29%) grew follicles to > 14 mm that regressed

When OC were initiated at 18 mm, 14/15 women (93%) ovulated and 1 woman (7%) developed a hemorrhagic anovulatory follicle. Four of the women that ovulated in the 18 mm treatment group developed 2 follicles $\geq$ 10 mm. The additional follicles ovulated (n=1), regressed (n=2) or developed into a hemorrhagic anovulatory follicle (n=1).

The diameter profiles of all follicles that grew to $\geq$ 10 mm after OC use, and corresponding concentrations of serum estradiol-17ß and LH, are shown for each treatment group in Figures 8.1-8.3. Follicle growth was associated with a rise in estradiol-17ß and LH levels in all treatment groups. Once estradiol and LH reached peak concentrations, follicle diameter continued to increase for 0-5 days at which time the follicle either ovulated, regressed or became cystic. The only exceptions to these observations were the 3 women in the 14 mm group that developed anovulatory follicular cysts. In these 3 cases, follicle growth continued for 6-10 days after peak

concentrations of LH and estradiol were observed. Anovulatory follicular cysts grew larger than any other follicles observed in the study (Table 8.1, $p<0.05$).

Mean values for peak follicle diameter and peak concentrations of estradiol-17ß, LH and progesterone within and among each treatment group compared to the reference group are shown in Table 8.1. Overall peak concentrations of estradiol, LH and progesterone were lowest in the 10 mm group, moderate in the 14mm group, and greatest in the 18 mm group ($p<0.05$). Peak concentrations of estradiol, LH and progesterone were lower in all treatment groups compared to the reference group ($p<0.05$).

Peak estradiol levels were greater in follicles that developed to > 14 mm compared to those that grew to 10-14 mm ($p<0.05$). Peak concentrations of estradiol and LH were not different among follicles > 14 mm that ovulated and those that did not ($p>0.05$). Day 6 progesterone was > 5 ng/mL (i.e., normal post-ovulatory levels) in all cases when ovulation was observed. Day 6 progesterone concentrations were greater than day 9 concentrations in all groups where ovulation occurred ($p<0.05$).

Changes in endometrial thickness and pattern during the study period are shown in Figure 8.4. No differences in the peak endometrial thickness and pattern were observed among the 14 mm, 18 mm and reference groups ($p>0.05$). However, peak endometrial thickness and pattern were lower in women randomized to the 10 mm group compared to the reference group ($p<0.05$).

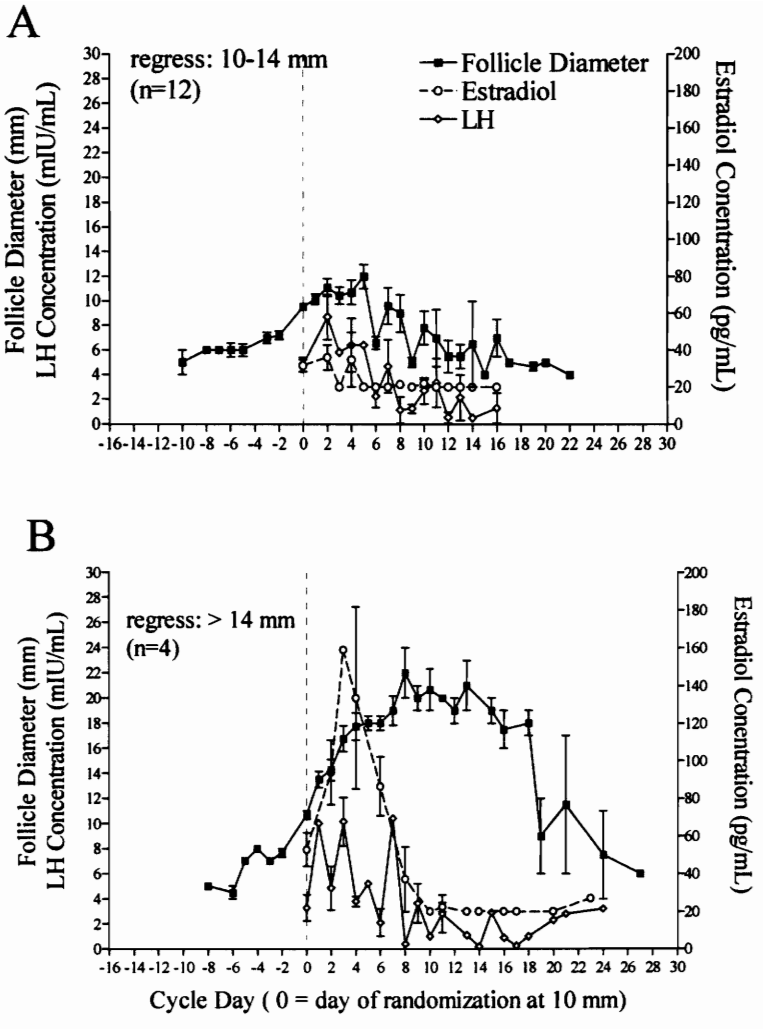

Figure 8.1: Follicle diameter profiles and serum concentrations of estradiol-17β and LH for women randomized to the initiate OC use at 10 mm. Follicles which grew to 10-14 mm and then regressed (A) and grew to > 14 mm and then regressed (B) are shown separately. Data are centralized to the day of randomization at 10 mm.

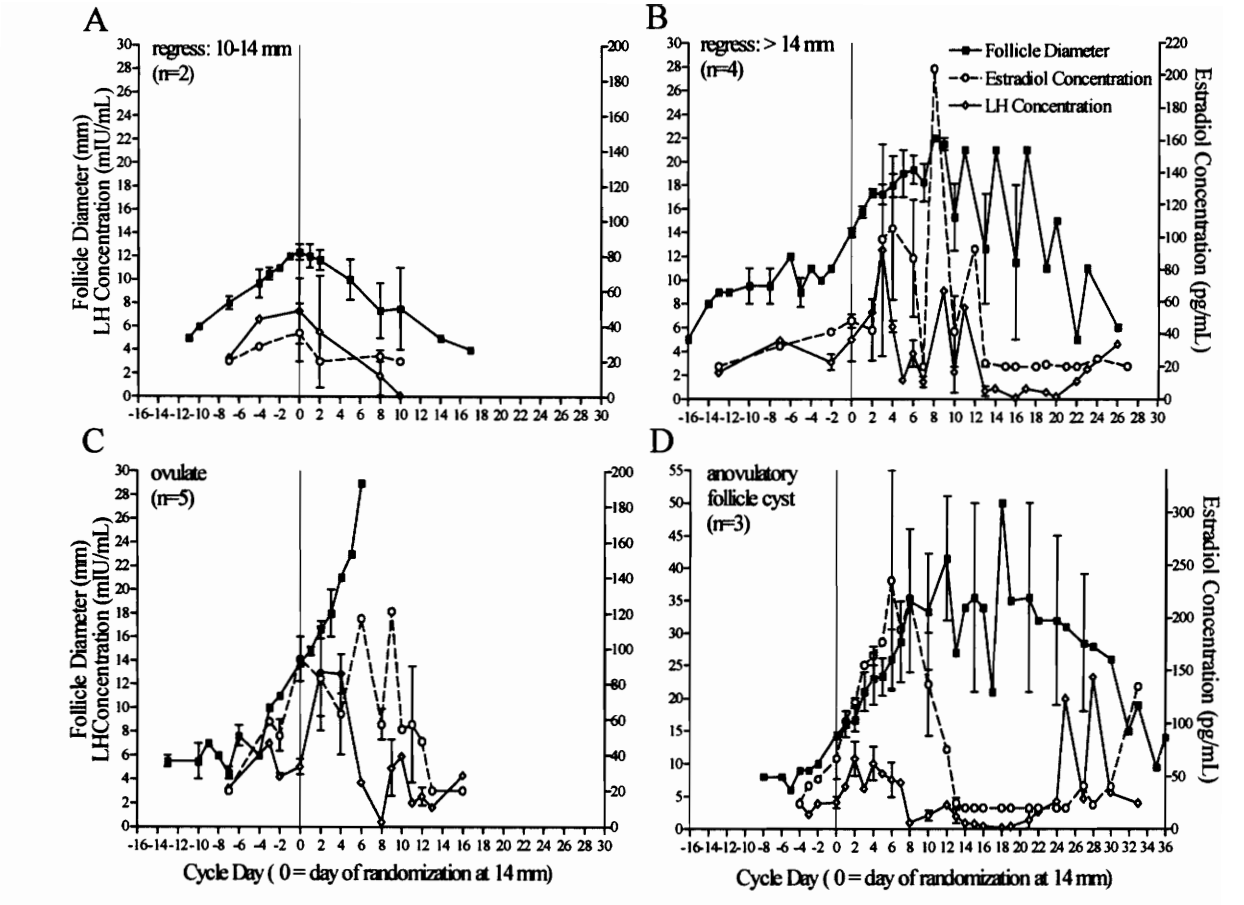

Figure 8.2: Follicle diameter profiles and serum concentrations of estradiol-17β and LH for women randomized to initiate OC at 14 mm. Follicles which grew to 10-14 mm and then regressed (A), grew to > 14 mm and then regressed (B), ovulated (C) or developed into anovulatory follicular cysts are shown separately (D). Data are centralized to the day of randomization at 14 mm.

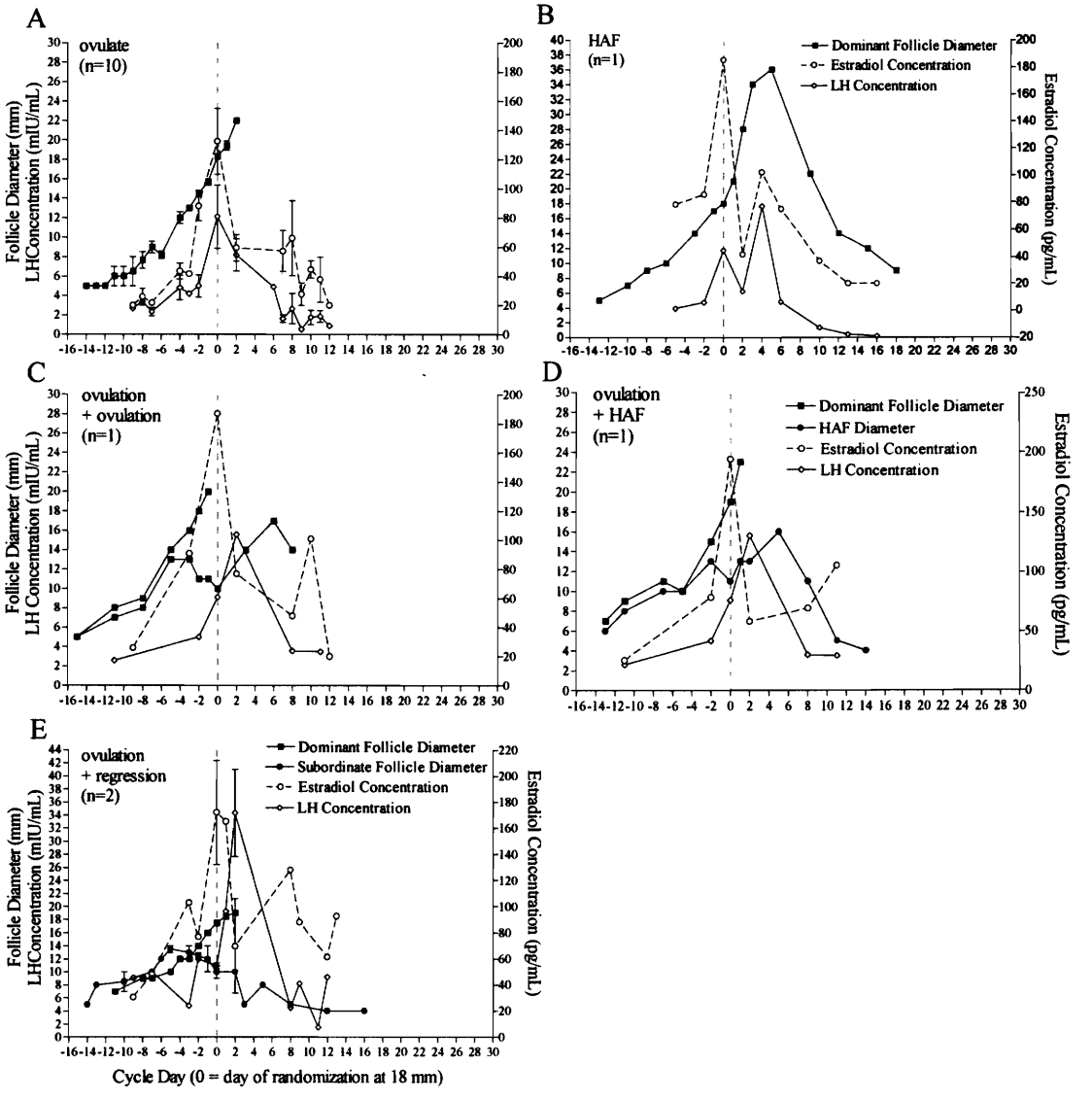

Figure 8.3: Follicle diameter profiles and serum concentrations of estradiol-17β and LH for women randomized to initiate OC at 18 mm. Follicles which ovulated (A), formed a HAF (B), ovulated *(follicle 1)* and ovulated *(follicle 2;* C), ovulated *(follicle 1)* and formed a HAF *(follicle 2;* D), and ovulated *(follicle 1)* and regressed *(follicle 2;* E) are shown separately. Data are centralized to the day of randomization at 18 mm.

Table 8.1: Descriptive statistics for follicle and endocrine endpoints within and among treatment and reference groups. All comparisons are within columns. Values with different superscripts indicate differences ($p<0.05$)

Group	Follicular Fate	N	Peak Diameter (mm)	Peak E_2 (pg/mL)	Peak LH (mIU/mL)	Day 6 P_4 (ng/mL)
10mm	Overall	16	13.1 ± 1.2^{a}	68.4 ± 14.9^{a}	8.0 ± 1.1^{a}	0.6 ± 0.04^{a}
	Regress: 10-14mm	12	10.7 ± 0.5^{o}	40.0 ± 6.5^{o}	7.9 ± 1.4^{o}	0.6 ± 0.1^{o}
	Regress: >14mm	4	20.5 ± 1.3^{p}	153.6 ± 27.9^{p}	8.3 ± 1.8^{o}	0.6 ± 0.1^{o}
14mm	Overall	14	$21.2\pm2.8b^{c}$	131.3 ± 22.7^{b}	11.2 ± 1.7^{b}	2.4 ± 0.8^{b}
	Regress: 10-14mm	2	12.5 ± 0.5^{q}	36.4 ± 16.4^{q}	7.4 ± 2.9^{q}	0.6 ± 0.1^{qr}
	Regress: >14mm	4	18.5 ± 1.5^{r}	119.4 ± 32.6^{r}	9.9 ± 4.0^{qr}	0.7 ± 0.0^{q}
	Anovulatory cyst	3	34.0 ± 10.1^{s}	238.3 ± 50.8^{s}	11.7 ± 1.8^{qr}	0.4 ± 0.2^{r}
	Ovulate	5	19.2 ± 2.5^{r}	114.6 ± 23.0^{r}	13.8 ± 3.2^{r}	5.3 ± 1.5^{qr}
18mm	Overall	15	19.8 ± 0.4^{b}	161.1 ± 11.8^{b}	16.4 ± 2.8^{c}	6.4 ± 0.9^{c}
	HAF	1	22.0	185.0	17.6	1.4
	Ovulate	10	19.5 ± 0.3^{t}	150.7 ± 15.9^{t}	14.1 ± 2.8^{t}	6.2 ± 1.1^{t}
	Ovulate + HAF	1	23.0	194.0	15.6	8.8
	Ovulate + Ovulate	1	20.0	187.0	3.7	6.7
	Ovulate + Regress	2	19.5 ± 0.5^{t}	172.0 ± 40.0^{t}	34.4 ± 6.7^{u}	8.8 ± 2.2^{t}
Reference	Ovulate	12	21.0 ± 0.3^{c}	204.2 ± 17.6^{c}	36.1 ± 5.6^{d}	11.6 ± 1.6^{d}

[a-d]: Overall comparisons between groups.
[o-p]: Comparisons within the 10 mm treatment group.
[q-s]: Comparisons within the 14 mm treatment group.
[t-u]: Comparisons within the 18 mm treatment group.

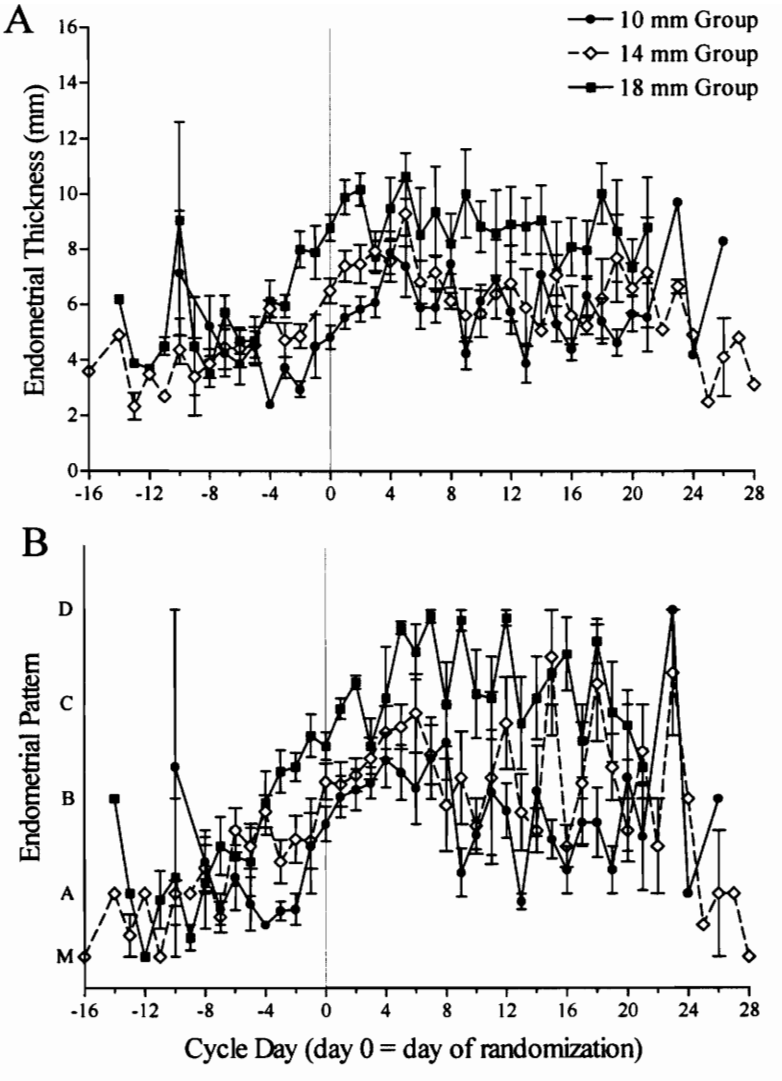

Figure 8.4: Profiles of endometrial thickness (A) and pattern (B) in women randomized to the receive OC at follicle diameters of 10, 14 and 18 mm. Data are centralized to the day of respective randomization.

8.5 Discussion

Follicular development, endometrial growth and endogenous endocrine levels were not suppressed effectively when OC use was initiated at mid to late stages of follicle development (i.e., $\geq$ 10 mm). Our hypothesis that follicular atresia would be induced when OC was initiated at a diameter of 10 mm was supported. No ovulations were observed in women randomized to the 10 mm group; rather, all follicles underwent regression. Our hypothesis that ovulation would occur when OC was initiated at a diameter of 14 mm was partially supported. Thirty-six percent of follicles that developed in the 14 mm group ovulated, while the remaining follicles regressed (43%) or formed anovulatory follicular cysts (21%). The hypothesis that ovulation would occur when women initiated OC use at 18 mm was supported. The majority of follicles (93%) in the 18 mm group ovulated, and 1 woman (7%) formed a hemorrhagic anovulatory follicle. The development of anovulatory follicular cysts and hemorrhagic anovulatory follicles has been reported to occur in approximately 10% of normal menstrual cycles [62]. Therefore, the development of anovulatory follicular cysts and hemorrhagic follicles in the present study may not have been a result of OC use, but rather a naturally-occurring event.

Follicular development was not effectively suppressed when OC was administered at mid to late stages of follicle development, apparently because physiologic selection of dominant follicles had already occurred. We have previously reported that selection of dominant follicles during natural menstrual cycles occurred at a diameter of 10 mm [40]. Therefore, initiation of OC when follicles were < 10 mm may have more effectively suppressed the development and ovulation of follicles by inhibiting follicle selection. The inability to inhibit pre-ovulatory follicle growth in this study may also be related to the finding that it can take up to 7 days for combined OC to elicit maximum suppressive effects on follicle development [44].

Peak estradiol and LH levels were lowest in the 10 mm group (when all follicles regressed), moderate in the 14 mm group (when 36% of follicles ovulated and the remainder regressed or became cystic) and greatest in the 18 mm group (when 93% of follicles ovulated). Estradiol and LH levels were greater in follicles that developed to diameters > 14 mm compared to those that did not. These results supported the notion

that granulosa cell estradiol production increased with growth of the dominant follicle following the selection process [49-52]. We further interpreted our findings to support previous reports that the dominant follicle became increasingly responsive to LH as it continued to develop after selection [49-51].

Overall peak estradiol and LH concentrations were lower in all treatment groups compared to the reference group. We concluded that administration of OC interfered with the ability of dominant follicles to produce estradiol and respond to increasing concentrations of LH. Mean peak LH concentrations were below the clinically-accepted mid-cycle standards in all of the treatment groups. It therefore appeared that the LH surge was inhibited in all treatment groups. We found these results perplexing because ovulation occurred in 29% of women who initiated OC use at 14 mm and 93% of women who initiated OC use at 18 mm. It is likely that OC may have inhibited peak LH concentrations below normal standards. However, follicles at advanced stages of development may have been able to ovulate in response to the blunted LH levels. Discrepancies between pre-ovulatory LH levels in our study compared to clinical standards may also be related to recent findings that mean endocrine concentrations in women did not accurately reflect the individual peak concentrations [63]. We further argued that the previously derived clinically-accepted endocrine standards do not take into account the new knowledge of variability in patterns of follicle wave dynamics [40], time of ovulation [64] and endogenous endocrine levels [40] in women during natural menstrual cycles. Ovulations in the present study appeared to be normal, despite blunted LH levels. Pre-ovulatory follicles and corpora lutea were ultrasonographically indistinguishable from those previously documented in spontaneous menstrual cycles [40]. Furthermore, progesterone levels were > 5 ng/mL in all women that ovulated following OC use (i.e., above normal clinical luteal phase levels).

The hypothesis that endometrial growth would occur in association with ovarian follicular development, during hormonal suppression with OC, was supported. The endometrium developed to reference levels in association with pre-ovulatory follicle development and ovulation in the 14 and 18 mm groups. In contrast, the endometrium was suppressed below reference levels in association with follicular atresia in the 10

mm group. Our findings confirm previously held notions that the development of follicles during the follicular phase of the menstrual cycle is closely associated with development of the endometrial lining prior to ovulation [59].

The initiation of OC in the presence of a dominant follicle did not inhibit follicle growth, but rather allowed the dominant follicle to continue growing, in association with increasing concentrations of estradiol and LH. None of the follicles in the 10 mm group ovulated; however, follicles in the 14 and 18 mm group ovulated. Dominant follicles (i.e., $\geq$ 10 mm) have been detected within the first 7 days of the menstrual cycle [40]. Therefore, women who may initiate OC use after 7 days of unsuppressed follicle growth (i.e., "Sunday Start" regimen) have likely already selected a dominant follicle which may continue to develop and ovulate following OC initiation. Our findings compared with those of previous studies in which re-initiation of OC use after a 7 day HFI did not inhibit the development of dominant follicles, but rather allowed their continued development to pre-ovulatory and cystic diameters [32, 36, 44]. These data, taken together, provide rationale for discouraging the use of "Sunday Start" OC regimens and reducing or completely eliminating the HFI in OC regimens.

It is not currently known why some follicle ovulate during OC use while others regress or form follicle cysts. It did not appear that the LH and estradiol surges were inhibited in follicles that reached pre-ovulatory levels but failed to ovulate. There were no differences in peak LH and estradiol concentrations between ovulatory versus anovulatory follicles that developed > 14 mm (i.e., in the 14 mm treatment group). However, LH levels appeared to be blunted in all treatment groups compared to the reference group. These interesting results prompt further investigations to study the endocrine and ultrasonographic characteristics of anovulatory follicles. This knowledge would aid in determining the mechanisms underlying anovulatory infertility and the failure to respond to ovulation induction therapy.

Endometrial growth was not suppressed below reference levels when OC were initiated at follicle diameters of 14 and 18 mm (i.e., when ovulations occurred). However, the endometrium was suppressed below normal reference levels in women that initiated OC use at a diameter of 10 mm (i.e., when no ovulations were observed). It appeared that OC regimens acted primarily at the hypothalamus and pituitary to

inhibit endogenous gonadotropin secretion, secondarily at the level of the ovary to suppress follicular development and follicular estradiol production, which then elicited a tertiary suppressive effect on the endometrium. The mechanisms of action underlying emergency contraceptive (EC) formulations are not currently known. Future research should be performed to determine the effects of administering EC regimens at different stages of follicular development during the menstrual cycle. This information would also aid in our understanding of the effects of EC regimens on ovarian and uterine function.

In summary, we demonstrated that dominant follicles produced estradiol and responded to elevated concentrations of LH as they developed during the mid to late follicular phase of the menstrual cycle. Our findings supported previously reports of antral follicle development during the follicular phase [50,59]. In retrospect, it would have been useful to have included a third treatment group, in which women were administered OC prior to follicle diameters of 10 mm. The inclusion of this treatment group may have provided insight into mechanisms underlying the selection process, particularly the shift from FSH to LH sensitivity and increased estradiol and inhibin secretion that has been documented in dominant follicles of domestic animals [50]. The mechanisms underlying follicle development were evaluated only during the follicular phase of the menstrual cycle. However, the next step is to apply similar techniques to follicles that develop in anovulatory follicular waves during the luteal phase [40]. This information may allow us to determine why dominant follicles develop in the luteal phase of spontaneous menstrual cycles in some women but not others, and why dominant follicles in the luteal phase fail to ovulate.

8.6 Acknowledgments

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Chapter 9

GENERAL DISCUSSION

The ovary's role as the master organ of the female reproductive system necessitates its dynamic morphology. The ovaries function as a repository for female germ cells throughout a woman's reproductive lifetime. The ovary is also a hormonal workstation, whereby reproductive steroid and gonadotropin hormones are produced to regulate cyclic changes in ovarian and uterine function during the menstrual cycle. The study of ovarian follicular dynamics in women is of particular interest because the ovarian follicle is the basic structural and functional unit of the ovary. The ovarian follicle houses the oocyte as it undergoes meiotic maturation, releases the oocyte in the process of ovulation and transforms into the corpus luteum following ovulation. The follicle and resulting corpus luteum are responsible for responding to the secretion of pituitary hormones by producing estrogen and progesterone. The gonadotropins and ovarian steroids are ultimately responsible for regulating the entire series of events that result in the release of a fertilizable oocyte and the maintenance of a subsequent pregnancy. Follicles function as exocrine glands during the process of ovulation and endocrine glands during the secretion of reproductively active steroid hormones.

At present, there is a global demand for strategies to manipulate the development of follicles within the ovary. The number of women requiring contraception for family planning is increasing world-wide [1]. Paradoxically, there is also an increase in the number of women requiring treatment for female-factor infertility [2]. A greater understanding of the basic mechanisms underlying the development and ovulation of ovarian follicles will allow us to determine how reproductive function is altered under the influence of hormonal contraception. This information will allow the production of more safe and efficacious contraceptive

options for women. Knowledge about ovarian function during natural menstrual cycles, and under the suppressive effects of OC, aids in our ability to stimulate ovarian follicular development with exogenous gonadotropins comprising infertility therapies.

In the series of studies described in this thesis, we evaluated the growth and regression of ovarian follicles during natural menstrual cycles and oral contraception (OC) cycles. Specifically, we quantified changes in the numbers and diameters of follicles, detected ovulation and assessed changes in the growth and regression of corpora lutea using high-resolution transvaginal ultrasonography. Changes in follicular and luteal development were then correlated with changes in circulating concentrations of reproductively-active hormones and endometrial growth to provide a comprehensive approach to understanding ovarian and uterine function.

9.1. Ovarian Follicular Dynamics during Natural Menstrual Cycles

Over the past 50 years, a traditional theory of ovarian follicular development during the menstrual cycle has become generally accepted. According to this theory, a single cohort of antral follicles is recruited to grow in each ovary during the late luteal phase of the menstrual cycle, in association with rising FSH levels [3, 4]. A single dominant follicle is believed to be selected from this cohort for preferential growth in the early-mid follicular phase [5]. The selection process has been shown to occur in association with decreasing FSH levels [6]. Therefore, the follicle that is selected is believed to exhibit increased FSH sensitivity. The dominant follicle is believed to produce inhibin which suppresses the growth of subordinate follicles, and estradiol which induces the formulation of granulosa cell LH receptors. The dominant follicle is thought to become increasingly responsive to LH as it continues to develop and ovulate [7, 8]. After ovulation at mid-cycle, progesterone production from the CL is believed to inhibit the development of follicles during the luteal phase of the menstrual cycle [9-14].

We were prompted to challenge the traditional theory of human folliculogenesis, based on clinical observations of large antral follicles in the luteal phase of healthy reproductive-aged women. 'Waves' of follicle development have been well-documented in domestic animal species [15-18]. Follicle waves were defined as the

synchronous growth of a group of follicles at regular intervals during the estrous cycle. Two or three waves were most commonly observed during the bovine and equine estrous cycles. The final wave was ovulatory while preceding waves were anovulatory during the bovine estrous cycle [15]. Major and minor waves of follicle development have been further characterized during the equine estrous cycle [17]. Major waves were those in which selection of a dominant follicle occurred, and minor waves were those in which a dominant follicle failed to develop. Major waves were characterized as anovulatory or ovulatory major waves. The development of large follicles at more than one time during the menstrual cycle in women has been described as 'follicular wave activity' occasionally in the literature [19-22]. In most cases, however, it appeared that luteal phase follicles were believed to represent an abnormal reproductive event. In contrast, follicle 'waves' were also described in women as the continuous entry of pre-antral resting follicles into the growing phase throughout the menstrual cycle [23].

We documented for the first time that waves of antral follicular development occurred during the menstrual cycle of normal, healthy women (Chapter 3). Sixty-eight percent of 50 women studied exhibited 2 waves of follicle development during an interovulatory interval (IOI), while 32 % exhibited 3 waves. We further determined that major and minor waves of follicle growth occurred in women (Chapter 4). Major waves were defined as those in which a dominant follicle (i.e., $\geq$ 10 mm) was selected for preferential growth, and minor waves were those in which dominance was not manifest. The final wave of the IOI was a major ovulatory wave, while preceding waves were either major anovulatory waves or minor waves. We concluded that follicular development in women resembled that previously documented in the equine species [17].

Waves of follicular development in women occurred in association with changes in reproductively active hormones during the menstrual cycle. Follicle waves were preceded by a rise in circulating concentrations of FSH (Chapter 4). Women with 2 follicle waves exhibited an earlier rise in follicular phase estradiol levels compared to women with 3 follicle waves, presumably due to earlier emergence of the dominant follicle. Women with 2 versus 3 follicle waves also exhibited earlier pre-ovulatory surges in estradiol, LH and FSH, resulting in a shorter IOI (Chapters 3 and 4).

The development of anovulatory follicular waves prior to the ovulatory wave in the bovine species has been attributed to negative feedback effects of luteal progesterone production on LH secretion following ovulation [24, 25]. Luteal area and progesterone production remained higher for longer periods of time in animals with 3 versus 2 follicle waves [26, 27]. Anovulatory waves continued to develop until resolution of the CL, and the dominant follicle present at the time of luteolysis became the ovulatory follicle.

We did not detect an LH surge during the luteal phase of the menstrual cycle (Chapter 4), which may have explained why follicles that emerged during the luteal phase did not ovulate. We were unable to establish that elevated progesterone during the luteal phase was responsible for allowing anovulatory follicles to develop prior to the ovulatory wave (Chapter 5). No differences were detected in the lifespan of the CL in women with 2 and 3 follicle waves. The CL began to regress much earlier in women than in the bovine species, and the dominant follicle present at the time of luteolysis was not the follicle that eventually ovulated. The CL in women appeared to produce more progesterone during the luteal phase and remain larger during the follicular phase in women with 2 versus 3 follicle waves. It therefore appeared that the CL had a suppressive effect on the emergence of a third wave in women with 2 follicle waves. We also reported greater luteal phase serum progesterone levels and greater follicular phase luteal area in women that developed minor versus major waves during the luteal phase. The latter findings further supported our initial interpretations that the CL elicited a suppressive effect on follicle development.

Our elucidation that the CL inhibited the emergence of follicle waves in women is opposite to what has been documented in domestic animals (i.e., bovine species). We therefore believe that the CL may influence the development of follicular waves in a species-specific fashion. It is possible that the equine species may be a better model for studying follicular and luteal dynamics in women compared to the bovine species, given that selection of a dominant follicle occurs in some waves but not others in both women and mares. It is also possible, although unlikely in our opinion, that the mechanisms underlying follicle wave dynamics in women are entirely different from those in domestic animals. Continued research is required to elucidate whether the CL acts in a

species-specific manner to influence the ovarian follicular wave phenomenon. Future research should also be performed to elucidate the influence of luteal inhibin and estradiol secretion on follicle wave dynamics in women.

Endometrial development was more pronounced during the follicular phase in women with 2 versus 3 follicle waves (Chapter 6). The earlier development of the endometrial lining in women with 2 versus 3 follicle waves was associated with earlier emergence of the dominant ovulatory wave (Chapter 4). We concluded that earlier prroduction of estradiol from the dominant follicle during the follicular phase was responsible for a more developed endometrial lining in the peri-ovulatory period in women with 2 follicle waves.

The documentation of a follicular wave phenomenon in women has challenged the notions of human follicle dynamics that have prevailed over the past 50 years, and provided a new model for studying ovarian follicular development during the menstrual cycle. In early studies, indirect evaluations of ovarian and uterine function were made based on measurements of urine and serum reproductive steroid hormone concentrations, and sometimes the intermittent use of transabdominal ultrasonography. However, the development of high-resolution transvaginal ultrasonography has allowed more precise visualization of the ovaries and uterus. The use of transvaginal ultrasonography, in concert with measurements of serum sex steroid and gonadotropin concentrations, allowed us to serially characterize the structural and functional dynamics of follicle development during the menstrual cycle.

Our newly-acquired knowledge of ovarian follicular waves in women has scientific implications for the future study of ovarian biology, as well as clinical implications for the development of new contraceptive and infertility therapies. A great deal of variability has been reported to exist in the levels of reproductively-active steroid hormones and gonadotropins during the menstrual cycles of healthy, reproductive-aged women [28]. It is likely that this variability is due to the different patterns of follicle dynamics that we documented in women. We expect that documentation of ovarian follicular waves will have ramifications for the use of clinical tests that measure steroid and gonadotropin hormone levels to determine the reproductive status of women (eg. day 3 FSH to detect peri-menopause).

Documentation of follicular waves will also allow more accurate determination of the time of spontaneous ovulation. The knowledge that cohorts of antral follicles develop at regular intervals during the menstrual cycle may provide more opportunities for stimulating ovarian follicular development prior to ovulation induction with or without oocyte retrieval for the treatment of infertility. The duration of infertility treatment would decrease, reducing the financial burden to patients and our health care system. In addition, patient waiting lists for infertility therapy would be reduced by the ability to more effectively synchronize follicle stimulation schedules among women.

We expect that the ovarian follicular wave phenomenon in women will also have implications for the development of new hormonal contraceptive options. A greater understanding of follicular waves during spontaneous menstrual cycles allows us to understand the mechanisms underlying hormonal contraceptive regimens. This information will aid in the development of safer and more efficacious contraceptive formulations. Emergency contraception (EC) is becoming widely-used among women of reproductive age, and is now available as an 'over the counter' contraceptive. The most commonly used EC regimens are the Yuzpe regimen (i.e., Levonorgestrel in combination with Ethinyl Estradiol) and the Plan B regimen (i.e., Levonorgestrel alone). Emergency contraceptives have been shown to prevent pregnancy when taken within 72 hours of unprotected intercourse followed by a second dose 12 hours later [29-32]. However, the mechanisms of action of EC are not fully understood. It has been speculated that EC regimens may function to suppress follicle development and ovulation when administered during the late follicular phase of the cycle, and disrupt endometrial development and the potential implantation of a conceptus following ovulation [33-37]. The possibility that EC may interfere with implantation has raised concern by some members of the scientific, medical and lay communities. However, knowledge that follicles develop at regular intervals during the menstrual cycle provides rationale for the notion that EC may function primarily at the level of the hypothalamus and pituitary to inhibit gonadotropin secretion, secondarily at the ovary to inhibit follicle development, and thirdly at the endometrium, when administered at any given stage of the menstrual cycle. Research is required to confirm or refute this theory.

We have come a long way in our understanding of follicle development during the human menstrual cycle. However, there is still a great deal to learn about this complex physiologic process. The precise mechanisms underlying physiologic selection of dominant follicles in women have yet to be determined. The role of growth factors in regulating ovarian follicular waves in women is not known. In addition, animal research is currently underway to elucidate the genes expressed during the development, regression and ovulation of ovarian follicles.

We evaluated follicle growth and regression over the course of one IOI. It would be useful to know whether the patterns and numbers of follicle waves were consistent over subsequent cycles. The dynamics of early antral follicle development (i.e., < 4 mm) are not currently known. It is possible that early antral follicles may develop continuously during the menstrual cycle, or in a wave-like fashion. We anticipate that advancements in ultrasonographic imaging capabilities over the next several years will allow us to visualize follicles, and the oocytes they contain, at earlier stages of development. Computer-assisted ultrasonographic image analyses could be used to determine image attributes of dominant versus subordinate follicles in women, as previously documented in the bovine species [38]. Image analyses could be used to evaluate the image attributes of dominant follicles in ovulatory versus anovulatory waves, as well as follicles in major versus minor waves. In addition, animal models could be used to evaluate the competence of oocytes within dominant follicles from anovulatory versus ovulatory waves. Oocyte competence could then be correlated with follicular and luteal data. Information about the physiologic and biochemical relationships between follicle development and oocyte competence would aid in determining the functional status of follicles (i.e., growth or atresia), the ovulatory potential of follicles and the fertilization capacity of oocytes during the estrous and menstrual cycles.

9.2 Ovarian Follicular Dynamics during the Use of Oral Contraception

The evaluation of ovarian follicle development in women using OC is a vital component in determining contraceptive efficacy. Combined OC regimens contain exogenous estrogen and progestin which are believed to provide negative feedback on

hypothalamic GnRH secretion, which in turn inhibits pituitary FSH and LH secretion to suppress follicle growth and prevent ovulation [39-44]. The mechanisms underlying the suppression of the hypothalamo-pituitary-ovarian axis during the use of OC are not fully understood. Improvements in our abilities to image the ovaries over the past 30 years have allowed researchers to determine that follicle activity is not completely suppressed during OC use [45: review]. Reports on follicle activity and ovulation during OC use have been variable. Some researchers have failed to detect ovulation during OC use [46-61], while others have documented the development of dominant follicles, ovulation and pregnancy [62-70]. Discrepancies in results could be attributed to different methods used to assess follicle development and ovulation. In early studies, urine or serum endocrine levels (i.e., LH and progesterone) were measured to indirectly evaluate the process of ovulation. In more recent studies, infrequent transabdominal and transvaginal ultrasonography, with or without assessment of endocrine levels, has been used to monitor ovarian follicular growth and ovulation during OC use. Considering the variability in reproductive hormone levels, follicle wave dynamics and day of ovulation in women during the menstrual cycle [28, 71], we believe that accurate determination of the mechanisms of action of OC requires frequent and comprehensive ultrasonographic and endocrinologic examination. Few studies of this nature, however, have been performed.

The development of large antral follicles during OC use has often been attributed to user non-compliance [72]. Specifically, women who missed doses, switched incorrectly from one formulation to another, or took medications that interfered with OC metabolism were found to be at a greater risk of ovulating and conceiving. We used high-resolution transvaginal ultrasonography to serially track the identities of individual dominant follicles that developed during the compliant use of 3 different OC regimens (Chapter 7). Follicles developed to ostensibly ovulatory diameters in women taking all 3 OC regimens. Follicle development to diameters $\geq$ 14 mm during OC use was associated with changes in estradiol which were comparable to those observed during natural menstrual cycles (Chapter 4). In addition, endometrial development occurred to levels similar to that previously documented during spontaneous menstrual cycles (Chapter 6). Surprisingly, however, no ovulations were

observed during the study. Transvaginal ultrasonography and measurements of serum estradiol and progesterone concentrations were also used to evaluate follicle development during the use of OC versus an experimental transdermal contraceptive device (Appendix). Oral and transdermal contraceptive efficacy was evaluated during standard dosing and following dosing errors. Pre-ovulatory follicle development and ovulations were observed in women administered both oral and transdermal contraceptive regimens in this study. The incidence of follicle activity and ovulation was greater in women using OC compared to women using the contraceptive patch. The development of follicles $\geq$ 12 mm and incidence of ovulation did not appear to differ during proper dosing of oral and transdermal contraception compared to that following dosing errors. Based on the results of these studies, we concluded that follicle development during oral and transdermal contraceptive use was a common phenomenon, not associated with user non-compliance. In addition, the transdermal patch was proven to be a safe and efficacious method of suppressing ovarian function. It has recently been approved for use by the FDA and Health Canada.

There has been increasing evidence to indicate the degree of follicular activity observed in women taking OC depended upon the dose of estrogen comprising the OC formulation [48, 70, 73-75]. The results of our studies supported these notions (Chapter 7, Appendix). The number of dominant follicles observed and the maximum diameter of dominant follicles that developed were greater in women administered 20 μg EE formulations compared to 30 and 35 μg EE. In retrospect, it would have been useful to use OC formulations containing the same type and dose of progestin, to rule out the progestin as a confounding factor on the effect of OC on follicle growth. The effect of different progestins comprising OC on the incidence of follicle activity was not evaluated in these studies, and has yet to be accurately determined.

Follicle growth and ovulation has been observed during the 7-day HFI of OC regimens [45, 73, 76-79]. Likewise, endogenous FSH and estradiol levels at the end of the HFI have been reported to rise to levels which compared to those observed during the early follicular phase of the natural menstrual cycle [48, 79-81]. There has been an indication that shortening the HFI may reduce the incidence of follicle development during OC use [78, 82, 83]. On the contrary, missing the last dosing pills of a cycle

and/or the first dosing pills of the subsequent cycle, which extends the HFI, has been associated with a greater degree of follicle development [49, 77, 84-86]. Van Heusden et al. reported that small antral follicles (i.e., 4-5 mm) appeared to be capable of responding to FSH levels above the threshold for ovarian stimulation, even after prolonged periods of suppression with exogenous estrogen and progesterone [79]. Resumption of contraceptive medication at the end of the HFI decreased FSH levels irrespective of the presence of dominant follicles. If no dominant follicles developed during the HFI, suppression of follicular growth was maintained. If a dominant follicle developed during the HFI, follicle growth continued despite declining FSH concentrations. We were able to show that the vast majority (86%) of all dominant follicles that developed during compliant OC use emerged during the HFI (Chapter 7). We attributed the development of dominant follicles during the HFI to a loss of negative feedback mechanisms on endogenous FSH secretion, as previously reported [79]. Our results have implications for the development of new contraceptive methods. The finding that dominant follicles frequently developed during the HFI advocates for a reduction in the duration of the HFI or the use of continuous OC regimens in which the HFI is completely eliminated.

In the final study comprising this thesis, OC use was initiated at different stages of follicle development (Chapter 8). This study allowed us to test hypotheses about changes in follicle development and endocrine parameters that occurred during the follicular phase of the menstrual cycle. In addition, we increased our understanding about the mechanisms underlying OC use. The results of this study also provided insight into strategies for improving OC use.

We demonstrated that initiating OC use at mid to late stages of follicular development (i.e., $\geq$ 10 mm) did not suppress follicle development, endometrial growth and endogenous endocrine levels effectively. Initiating OC use at a follicle diameter of 10 mm induced atresia of dominant follicles. Initiating OC use at a diameter of 14 mm induced atresia, ovulation or the development of anovulatory follicular cysts. Ovulation was observed in the majority of follicles when OC use was initiated at a diameter of 18 mm. We concluded that OC did not suppress follicular development effectively because selection of dominant follicles for preferential growth had occurred by the time

OC use was initiated. These notions were supported by our earlier finding that selection of dominant follicles occurred at a diameter of 10 mm (Chapter 4). Therefore initiation of OC at diameters $\geq$ 10 mm, after follicles had been selected for preferential growth, allowed continued follicular development. In this study, we determined the effect of OC on follicle development $\geq$ 10 mm, which has been shown to represent the development of major waves during the menstrual cycle (i.e., waves in which selection of a dominant follicle has occurred, Chapter 4). It is worth mentioning, however, that some women may have exhibited minor waves prior to OC initiation. The effect of OC use on the development of minor waves is not known, and requires further investigation.

Initiating OC use at different stages of follicular development resulted in a differential response in the secretion of endogenous estradiol and LH. Peak estradiol and LH levels were lowest when follicle atresia occurred after initiating OC at a diameter of 10 mm, moderate when follicles regressed, ovulated or became cystic after OC use at 14 mm, and greatest when follicles ovulated following OC use at 18 mm. Peak estradiol and LH levels were also greater in women that developed follicles to diameters of > 14 mm compared to those that did not. These findings supported previous reports that dominant follicles were better able to secrete estradiol and respond to increasing concentrations of LH as a consequence of the selection processs [5, 8, 88]. The finding that the incidence of ovulation was directly proportional to the concentration of LH observed supported previous reports that LH is responsible for inducing ovulation of dominant follicles [87].

Ovulations that occurred following OC initiation appeared ultrasonographically indistinguishable from those observed during natural menstrual cycles (Chapter 3). Ovulations after OC use, however, were accompanied by mid-cycle LH levels below normal reference levels. We interpreted these findings to mean that OC inhibited peak levels of LH. However, follicles at advanced stages of development appeared to be able to ovulate in response to the blunted LH levels. It is also important to recognize that continued follicle development, estradiol and LH secretion and ovulation following OC initiation may also be related to the recent findings that combined OC can take up to 7 days to elicit maximal suppressive effects [79].

The development of dominant follicles to ostensibly ovulatory diameters (i.e., > 14 mm) when OC use was initiated at ≥ 10 mm has implications for the use of delayed OC start schemes (eg. "Sunday Start" regimens), in which OC initiation can be postponed until 7 days following menses. We demonstrated that dominant follicles have been selected prior to day 7 of spontaneous menstrual cycles (Chapter 4). Initiation of OC use in the presence of a dominant follicle did not suppress follicle development, but rather allowed continued development to pre-ovulatory and cystic diameters. We therefore concluded that women using delayed OC initiation schemes were at a greater risk of developing pre-ovulatory follicles and ovulating. We further concluded that initiating OC use on the first day of menses is the most effective method of suppressing follicle development in the first month of OC use. Our finding that follicle development, endometrial growth and endocrine secretion were not suppressed following the initiation of OC use at mid to late stages of follicle development provided insight into the mechanisms of action underlying OC use. Endometrial development was suppressed below levels observed during natural menstrual cycles in association with follicle regression when OC were initiated at a follicle diameter of 10 mm. However, the endometrium was not suppressed in association with ovulation and the development of follicular cysts following initiation of OC use at 14 and 18 mm. We interpreted these results to mean that OC elicited their effects first at the level of the hypothalamus and pituitary to inhibit gonadotropin secretion, and secondarily at the ovary to suppress follicle growth. In doing so, follicular estradiol production was inhibited which elicited a tertiary suppressive effect on the endometrium. The results of this study provide rationale for performing similar studies to evaluate the effect of emergency contraceptive regimens on follicular and endometrial development when administered at different stages of the menstrual cycle.

Future studies to evaluate follicular development during the use of OC should be focused on determining why some follicles ovulate during OC use and others do not. Peak LH and estradiol concentrations were not different between follicles that developed to > 14 mm and ovulated versus those that failed to ovulate. This information suggested that anovulation does not result from inadequate estradiol and LH stimulation. It is possible that anovulation may be related to the inability of follicles

to respond to increasing LH levels. Research is required to test this theory. Information about the ability of follicles to respond to increasing concentrations of LH during development would increase our understanding of the mechanisms underlying anovulatory infertility and failure to respond to ovulation induction therapy.

Additional studies are required to determine the effect of continuous OC use on follicle wave dynamics in women. The finding that the majority of dominant follicles that developed over 3 months of OC use emerged during the HFI substantiates continued research in this area. It seems likely that the incidence of follicle development would be significantly less if the HFI were completely omitted. However, it is also important to consider the incidence of break-through bleeding and patient satisfaction, as well as patient safety.

There is little known about how EC works; however, many women are relying on this contraceptive option to prevent pregnancy. Continued research is required to evaluate the effect of emergency contraceptive regimens administered at different stages of follicle development during the menstrual cycle. Knowledge about mechanisms underlying EC use would allow us to determine whether this method functions to interfere with embryo implantation and maintenance of pregnancy. Work in this area will be critical for providing women with the safest and most effective contraceptive options.

9.3 Overall Conclusions

The studies described in this thesis provided detailed information about ovarian follicular development during natural menstrual cycles and oral contraceptive cycles.

The following 8 hypotheses were supported:

1) Women exhibit 2 and 3 waves of follicle development during the menstrual cycle, comparable to those previously described in domestic animal species.
2) Women exhibit major and minor patterns of follicle wave dynamics during the menstrual cycle, resembling ovarian follicular activity in the equine species.
3) The emergence and development of ovarian follicular waves in women appear to be negatively influenced by the secretion of progesterone from the corpus luteum.
4) The development of 2 versus 3 follicular waves during the menstrual cycle is associated with differential changes in endometrial development.
5) Ovarian follicles develop to ostensibly ovulatory diameters during the compliant use of oral contraception.
6) Ovarian follicular development during oral contraceptive use occurs due to loss of endocrine suppression during the hormone-free interval.
7) The degree of follicular activity observed during oral contraceptive use depends on the dose of estrogen comprising the oral contraceptive regimen.
8) Ovarian follicular development is not effectively suppressed when OC use is initiated at mid to late stages of follicle development.
9) Dominant follicles produce estradiol and become increasingly responsive to LH as they develop during the follicular phase of the menstrual cycle.

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Appendix

ORTHO EVRA™ VERSUS ORAL CONTRACEPTIVES: FOLLICULAR DEVELOPMENT AND OVULATION IN NORMAL CYCLES AND AFTER AN INTENTIONAL DOSING ERROR

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A1. Abstract

Objective: To compare the effects of the contraceptive patch to oral contraceptives (OCs) on follicular size and incidence of ovulation in normal cycles and after dosing errors.

Design: Randomized, open-label

Setting: 12 centers

Patients: 124 ovulatory women

Interventions: Subjects received either the patch (Groups 1 or 2) or 1 of 3 OCs. Correct dosing occurred in Cycles 1, 2, 3, and 5. The following dosing errors were planned during Cycle 4, a shortened 10-day cycle: 1) patch Group 1 subjects wore 1 patch for 10 consecutive days; 2) patch Group 2 and OC subjects, 7 dosing days were followed by 3 drug-free days.

Main outcome measure: Follicular size, as determined at each cycle by the maximum mean follicular diameter

Results: After a 3-day dosing error, follicular size was significantly smaller in the patch group (mean of 7.0 mm) versus each OC group (range of means: 11.8–17.1 mm) ($p<0.05$ for all comparisons of patch to OC). Similar results were seen after proper dosing. The incidence of ovulation was significantly lower for the patch users than for women using OCs.

Conclusions: Follicular size and incidence of ovulation were significantly reduced among contraceptive patch users compared with women using OCs in normal cycles and after planned dosing errors.

A2. Introduction

Combination oral contraceptives (OCs) are the most commonly used form of reversible contraception.(1) While highly effective when used correctly, inconsistent or less than perfect compliance with daily dosing of OCs are major contributors to the higher contraceptive failure rates reported during typical use.(2,3) One study reported that 19% of OC users miss 1 or more pills per cycle,(4) while another found that 47% of OC users miss 1 or more pills, and 22% miss 2 or more pills per cycle.(5) Dosing errors have the potential to reduce the efficacy of a hormonal contraceptive agent. Missing 1

or more pills per cycle has been estimated to result in a 2.6-fold greater risk of unintended pregnancy when compared with women who use OCs correctly.(4)

A contraceptive patch (Ortho Evra™, Johnson & Johnson Pharmaceutical Research & Development, L.L.C., Raritan, NJ), which contains the progestin norelgestromin (previously known as 17-deacetylnorgestimate) and the estrogen ethinyl estradiol (EE), has been developed recently. The 20-cm^2 matrix patch is a thin, laminated, matrix-like system consisting of 3 layers: an outer protective layer of polyester; a medicated, adhesive middle layer; and a clear, polyester, release liner that is removed prior to patch application. The patch is applied on any of 4 anatomic sites (buttock, upper outer arm, lower abdomen, upper torso [excluding breast]). (Johnson & Johnson Pharmaceutical Research & Development, L.L.C., Raritan, NJ). The patch is applied on the same day each week for 3 consecutive weeks (21 days) followed by 1 patch-free week per cycle.

The primary objectives of the study reported here were to compare the effect of the contraceptive patch versus 3 OCs with regard to follicular size and the incidence of ovulation in normal cycles and following a planned dosing error. Secondary objectives were to compare the endocrine effects and safety profiles of these regimens.

A3. Materials and Methods

I. Study design

This was an open-label, parallel-group, 5-cycle study conducted at 7 centers in Canada and 5 centers in the United States. The protocol was approved by the institutional review board or ethics committee at each center, and the study was conducted in compliance with the regulations governing good clinical practice. After giving informed consent, subjects were randomized to the contraceptive patch (Groups 1 or 2) (a 20-cm^2 patch designed to deliver norelgestromin 150 μg and EE 20 μg daily to systemic circulation), a triphasic levonorgestrel (LNG) OC (Triphasil®; oral LNG 50 μg plus EE 30 μg for Days 1 to 6, LNG 75 μg plus EE 40 μg for Days 7 to 11, LNG 125 μg plus EE 30 μg for Days 12 to 21, and placebo for Days 22 to 28; Wyeth-Ayerst Pharmaceuticals, Philadelphia, PA), a monophasic LNG OC (Alesse®; oral LNG 100 μg plus EE 20 μg for Days 1 to 21, placebo for Days 22 to 28; Wyeth-Ayerst

Pharmaceuticals, Philadelphia. PA), or a triphasic norgestimate (NGM) OC (Ortho Tri-Cyclen®; oral NGM 180 μg plus EE 35 μg for Days 1 to 7, NGM 215 μg plus EE 35 μg for Days 8 to 14, NGM 250 μg plus EE 35 μg for Days 15 to 21, and placebo for Days 22 to 28; Ortho-McNeil Pharmaceutical, Inc., Raritan, NJ).

Correct dosing was planned for all subjects in Cycles 1, 2, 3, and 5. During these cycles, patch treatment was 3 consecutive 7-day patches (21 days) followed by 1 patch-free week. OC treatment for Cycles 1, 2, 3, and 5 was 1 pill daily for 21 consecutive days followed by placebo for 1 week. An intentional dosing error was planned in Cycle 4, a 10-day cycle in which proper dosing was not followed for Days 8 to 10. For the contraceptive patch Group 2 and OC subjects, 7 dosing days were followed by 3 drug-free days. The contraceptive patch Group 1 subjects wore 1 patch for 10 consecutive days to simulate a forgotten patch change. Cycle 5 was initiated immediately after the dosing error interval, thereby simulating the instructions for dosing error correction for the patch and OCs.

Study population

One hundred twenty-four healthy women between 18 and 35 years of age were randomized and received study drug. Subjects had to be nonsmoking; have regular menstrual cycles; have no evidence of cervical dysplasia; have a negative serum β-human chorionic gonadotropin (β-hCG) pregnancy test within 7 days prior to taking study drug; have at least 1 normal menses since the removal of an intrauterine device, if applicable; have seated systolic/diastolic blood pressure less than 140/90 mm Hg; agree to use only nonhormonal contraceptives, except the assigned study drug, for 2 months prior to randomization through the final study visit; and provide written informed consent. All subjects were provided with barrier methods of contraception (i.e., condoms) during the trial. Exclusion criteria included lactation or pregnancy within 6 months of study admission; any disorders that were contraindications to steroid hormonal therapy; Papanicolaou smear evidence of squamous intraepithelial lesions (low or high grade) or adenocarcinoma or other malignancy; history or presence of dermal hypersensitivity in response to topical application; alcohol or substance abuse within 12 months of screening; receipt of steroid hormonal therapy within 3 months of

screening; receipt of Depo-Provera® within 6 months of screening; and receipt of any experimental drug, device, or hepatic enzyme-inducing drug within 30 days of screening.

II. Patch description and use

The contraceptive patch is a thin matrix patch that consists of 3 layers: an outer protective layer of polyester; a medicated, adhesive middle layer; and a clear, polyester, release liner that is removed prior to patch application. Subjects wore the patch on any of 4 anatomic locations: buttocks, upper outer arm, lower abdomen, or upper torso (excluding the breasts). New patches could be applied to sites near the patch that was removed, but not to the exact place as the preceding patch. Subjects could maintain their usual activities, including bathing and swimming, while wearing the patch, but were instructed not to apply oils, creams, or cosmetics on or around the area of patch placement. In the event of accidental patch detachment, a replacement patch was to be applied immediately and worn for the remainder of that week.

III. Study evaluations

Follicular size:

Ovarian ultrasonographic examinations were performed prestudy and during Cycles 1 through 5, as outlined in Table 1. During any ultrasonographic examination, if a follicle was found to be 12 mm or larger, an ultrasonographic examination was performed daily until the follicle either showed signs of suspected ovulation or was determined to be stable in size and appearance for 4 consecutive days after which the normal examination schedule was resumed. The follicular length and width in the transverse and sagittal planes were determined and the mean of these measurements for each follicle was calculated. Measurements of the 2 largest follicles in each ovary were recorded. From these many mean follicular diameters per subject per cycle, the largest (maximum) mean follicular diameter (MMFD) per cycle was selected for each subject and utilized as the main outcome measure for follicular size.

Table A1: Cycle Days That Scheduled Ultrasonographic Examinations Were Performed for Cycles 1 Through 5

	Days
Cycle 1	7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27
Cycle 2	5, 12, 19, 26
Cycle 3	3, 6, 9, 12, 15, 18, 21, 24, 27
Cycle 4	7, 8, 9, 10
Cycle 5	1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27

Ovulation

Ovulation in this study was evaluated by a 2-step criteria. The first step was the observance by ultrasonographic examination of the disappearance of a large (periovulatory) follicle. Subjects who met this criterion had blood drawn for progesterone assay 7 to 10 days after the follicle disappearance. Progesterone levels ≥ 3 ng/mL after follicle disappearance were considered to be evidence of ovulation.(6)

Endocrine profile

Blood samples were collected for the endocrine profiles at various times during the study; prestudy, between Days 18 and 21 of the subject's menstrual cycle during Cycles 1, 3, and 5 and, for those subjects who completed 5 cycles of therapy, within 2 weeks after Day 28 of Cycle 5. A final blood sample was collected between 4 to 6 weeks post-treatment. The purpose of the post-treatment ultrasonographic scan and blood sampling were to examine a potential return to fertility. Blood samples were analyzed by Covance Central Laboratory (Indianapolis, IN) for luteinizing hormone (LH), follicle-stimulating hormone (FSH), progesterone, and estradiol.

Patch adhesion

Patch replacement information was used to assess patch adhesion. The percentage of patches replaced for the reason "fell off" was summarized as patches that completely detached due to lack of adhesion.

Safety

Adverse events, both those reported by subjects and those observed by study center personnel, were collected throughout the study. Vital signs (blood pressure, pulse rate, and oral temperature) and body weight were determined prestudy and during Cycles 1, 3, and 5 (or at early withdrawal), and physical and gynecologic examinations were performed prestudy and at Cycle 5 (or at early withdrawal).

IV. Statistical methods

Analyses of variance were performed to analyze the follicular size, utilizing the MMFD for each subject at Cycle 5 (ie, after dosing errors) and also during Cycles 1, 2, and 3 (ie, after proper dosing). For each cycle, pairwise comparisons between treatments were made using least significant difference multiple comparison procedures. The contraceptive patch (Groups 1 and 2 combined) was compared to each of the 3 OCs, and each OC was compared with the other formulations. At Cycle 5, the 2 patch groups were also compared. All subjects who received study drug and who had measurements taken from ovarian ultrasonographic examinations at baseline and between Days 18 and 21 of their menstrual cycle for at least 1 cycle of treatment were considered evaluable for the follicular development analysis. A chi-square test for association was performed to compare the incidence of ovulation between treatments at Cycles 1, 2, 3, and 5. If there was a significant difference across treatments, pairwise comparisons between treatments were made using Fisher's exact test. All subjects who received study drug and who had data available were included in the analysis.

Analyses of variance were performed to analyze the mean change from baseline to Cycle 3 for each endocrine hormone level evaluated. Pairwise comparisons were made using the nonparametric Wilcoxon-Mann-Whitney test so that outliers would not unduly influence comparisons. The contraceptive patch (Groups 1 and 2 combined) was compared to each of the 3 OCs and the 3 OCs were compared with each other. All subjects who received study drug and who had data available at baseline and Cycle 3 were considered evaluable for the endocrine profile analysis. Mean changes from baseline to Cycle 5 and 4 to 6 weeks post-treatment were also calculated.

A4. Results

Demographic characteristics and disposition

The demographic characteristics of the 4 treatment groups were comparable (Table A2). Of the 136 subjects randomized, 12 subjects did not receive study drug (4 subjects in the contraceptive patch group, 3 subjects in each of the triphasic LNG OC and triphasic NGM OC groups, and 2 subjects in the monophasic LNG OC group). Of the 124 treated subjects, 111 (90%) completed the study. Subject choice was the most common reason for premature discontinuation (7 subjects in the contraceptive patch group and 1 subject each from the triphasic LNG OC, triphasic NGM OC, and monophasic LNG OC groups). In the contraceptive patch group, 1 subject withdrew due to protocol violation (pretherapy pregnancy), another subject withdrew on Day 9 of the study due to a reported left paratubal complex cyst, and a third subject withdrew due to reasons not reported. Three subjects received the contraceptive patch for less than 16 days and were therefore excluded from the follicular development and incidence of ovulation analyses. No subjects who received OCs were excluded from these analyses. All 124 subjects were included in the safety analyses.

Table A2. Demographic Characteristics (All Subjects Randomized and Treated)

Parameter	Contraceptive patch n = 52	Triphasic LNG OC n = 22	Monophasic LNG OC n = 25	Triphasic NGM OC n = 25
Age, years	26.5 (5.1)	26.2 (4.4)	25.6 (4.9)	27.0 (4.9)
Race, %				
W/B/A/O	90/0/4/6	86/9/0/5	88/12/0/0	92/4/4/0
Height, cm	164.8 (6.6)	162.8 (10.1)	165.0 (7.7)	164.6 (6.0)
Weight, kg	64.0 (10.9)	65.0 (11.7)	67.2 (13.9)	64.1 (12.3)

OC = oral contraceptive; W/B/A/O = White/Black/Asian/Other.
Values are means (standard deviations [SDs]) unless otherwise specified.

Follicular development

At Cycles 1, 2, and 3 (ie, after proper dosing), treatment with the contraceptive patch resulted in a smaller follicular size compared with all OCs ($p < 0.05$ for all comparisons) (Figure A1). The monophasic LNG OC group had a significantly greater follicular size at Cycle 1 compared with the triphasic NGM OC group. No other statistically significant differences were observed after proper dosing.

At Cycle 5 (i.e., after dosing errors), follicular size was smaller in each of the contraceptive patch groups compared with the triphasic LNG OC ($p < 0.05$), the monophasic LNG OC ($p < 0.001$), and the triphasic NGM OC ($p < 0.05$) groups. Patch Groups 1 and 2 did not differ significantly from each other (7.1 vs 6.8 mm, respectively). Figure A1 shows the results with the patch groups combined. Comparisons of the OCs with one another revealed a greater follicular size in the monophasic LNG group compared with each of the other OC groups ($p < 0.05$ for both comparisons). No other statistically significant differences were observed after dosing errors.

At Cycles 1, 2, 3, and 5, more subjects had a follicular size of less than 12 mm in the contraceptive patch group compared with the OC groups (Table A3).

Ovulation

The incidence of ovulation was lower for the contraceptive patch than for the OCs (Table A4). Differences were seen between the contraceptive patch and the triphasic LNG OC at Cycles 3 and 5 ($p < 0.01$ and $p < 0.05$, respectively), between the contraceptive patch and the monophasic LNG OC at Cycles 1, 2, 3, and 5 ($p < 0.05$, $p < 0.01$, $p < 0.01$, and $p < 0.05$, respectively), and between the contraceptive patch and the triphasic NGM OC at Cycles 2 and 3 ($p < 0.05$ for Cycles 2 and 3). The incidence of ovulation in each of the OC groups ranged from 0% for women receiving triphasic LNG OC in Cycle 1 to 28% for women receiving monophasic LNG OC in Cycle 3.

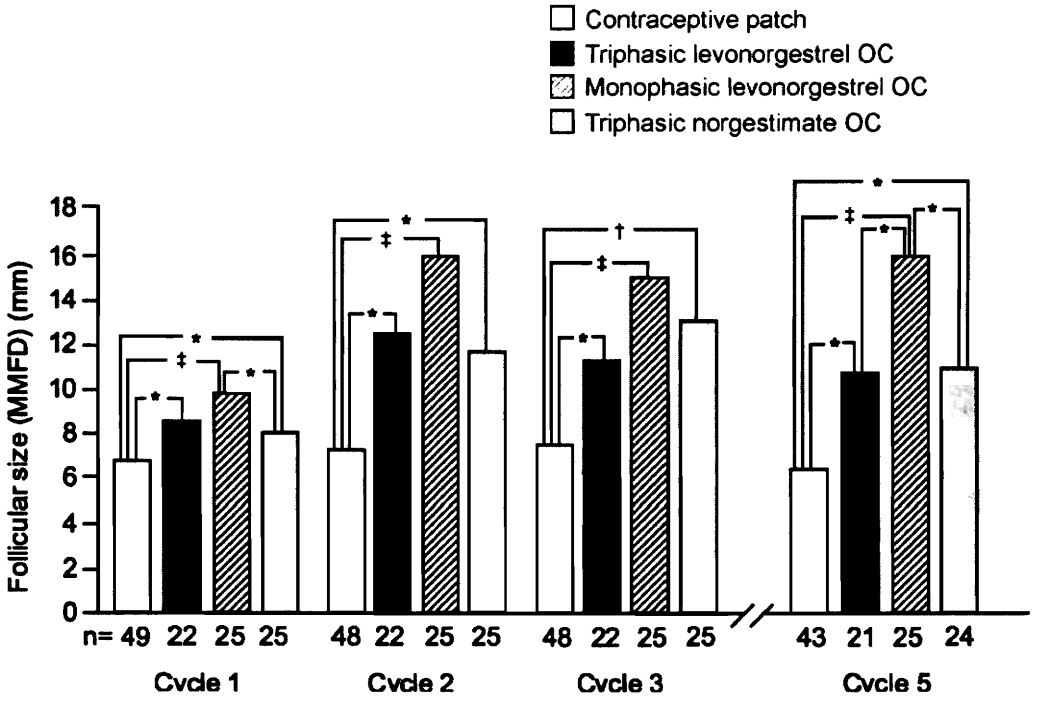

Figure A1. Follicular size (MMFD) at Cycles 1, 2, and 3 (i.e., after proper dosing) and at Cycle 5 (ie, after dosing errors). MMFD at Cycle 5 was measured from blood drawn on Cycle Days 18 to 21. $p < 0.05$; † $p < 0.01$; ‡p.001

Table A3: Frequency Distribution of Follicular Sizes (Maximum Mean Follicular Diameters [mm]) for All Evaluable Subjects in Properly Dosed Treatment Cycles

Follicular Diameter (MMFD)	Number (%) of subjects			
	Patch	Triphasic LNG OC	Monophasic LNG OC	Triphasic NGM OC
Cycle 1				
< 12 mm	48 (98)	20 (91)	19 (76)	23 (92)
12–20 mm	1 (2)	2 (9)	5 (20)	2 (8)
21–30 mm	0	0	1 (4)	0
> 30 mm	0	0	0	0
Cycle 2				
< 12 mm	46 (96)	16 (73)	12 (48)	19 (76)
12–20 mm	1 (2)	3 (14)	6 (24)	1 (4)
21–30 mm	1 (2)	1 (5)	5 (20)	4 (16)
> 30 mm	0	2 (9)	2 (8)	1 (4)
Cycle 3				
< 12 mm	48 (100)	16 (73)	12 (48)	18 (72)
12–20 mm	0	4 (18)	8 (32)	3 (12)
21–30 mm	0	1 (5)	4 (16)	1 (4)
> 30 mm	0	1 (5)	1 (4)	3 (12)
Cycle 5				
< 12 mm	42 (98)	15 (71)	16 (64)	18 (75)
12–20 mm	0	4 (19)	2 (8)	2 (8)
21–30 mm	0	1 (5)	3 (12)	3 (13)
> 30 mm	1 (2)	1 (5)	4 (16)	1 (4)

OC = oral contraceptive.
Percentages may not add to 100% because of rounding errors.

Table A4. Incidence of Ovulation (Serum Progesterone ≥ 3 ng/mL) (All Evaluable Subjects), Number Ovulating (%) in Properly Dosed Treatment Cycles

Cycle	Contraceptive patch	Triphasic LNG OC	Monophasic LNG OC	Triphasic NGM OC
1	0/49 (0)	0/22 (0)	3/25 (12)*	2/25 (8)
2	1/48 (2)	3/22 (14)	6/25 (24)†	4/25 (16)*
3	0/48 (0)	4/22 (18)†	7/25 (28)‡	4/25 (16)*
5	1/43 (2)	4/21 (19)*	5/25 (20)*	3/24 (13)

* $p < 0.05$ vs contraceptive patch.
† $p < 0.01$ vs contraceptive patch.
‡ $p < 0.001$ vs contraceptive patch.

Endocrine profile

Mean changes from baseline to Cycle 3 (ie, after proper dosing), from baseline to Cycle 5 (ie, after dosing errors), and from baseline to 4 to 6 weeks post-treatment are presented in Figure A2. All treatments resulted in mean decreases from baseline to Cycles 3 and 5 for the hormones that influence or indicate follicle development or ovulation (ie, LH, FSH, progesterone, and estradiol). Mean reductions from baseline to Cycle 3 were greater with the contraceptive patch versus the monophasic LNG OC for FSH ($p < 0.001$) and LH ($p = 0.027$); triphasic LNG OC reductions were greater compared with monophasic LNG OC for FSH ($p < 0.01$) and estradiol ($p = 0.05$); and triphasic NGM OC reductions were lower than monophasic LNG OC for FSH ($p = 0.015$). Mean changes from baseline to Cycle 5 (ie, after dosing errors) were generally similar to those observed at Cycle 3 (Figure 2). Mean LH and FSH concentrations returned to near baseline levels in all treatment groups at 4 to 6 weeks after treatment, with the exception of the LH concentration in the triphasic LNG OC group, which remained below baseline. In all treatment groups, mean progesterone and estradiol concentrations remained decreased from baseline at 4 to 6 weeks post-treatment, although the reduction was less than that observed at Cycles 3 or 5 (Figure A2).

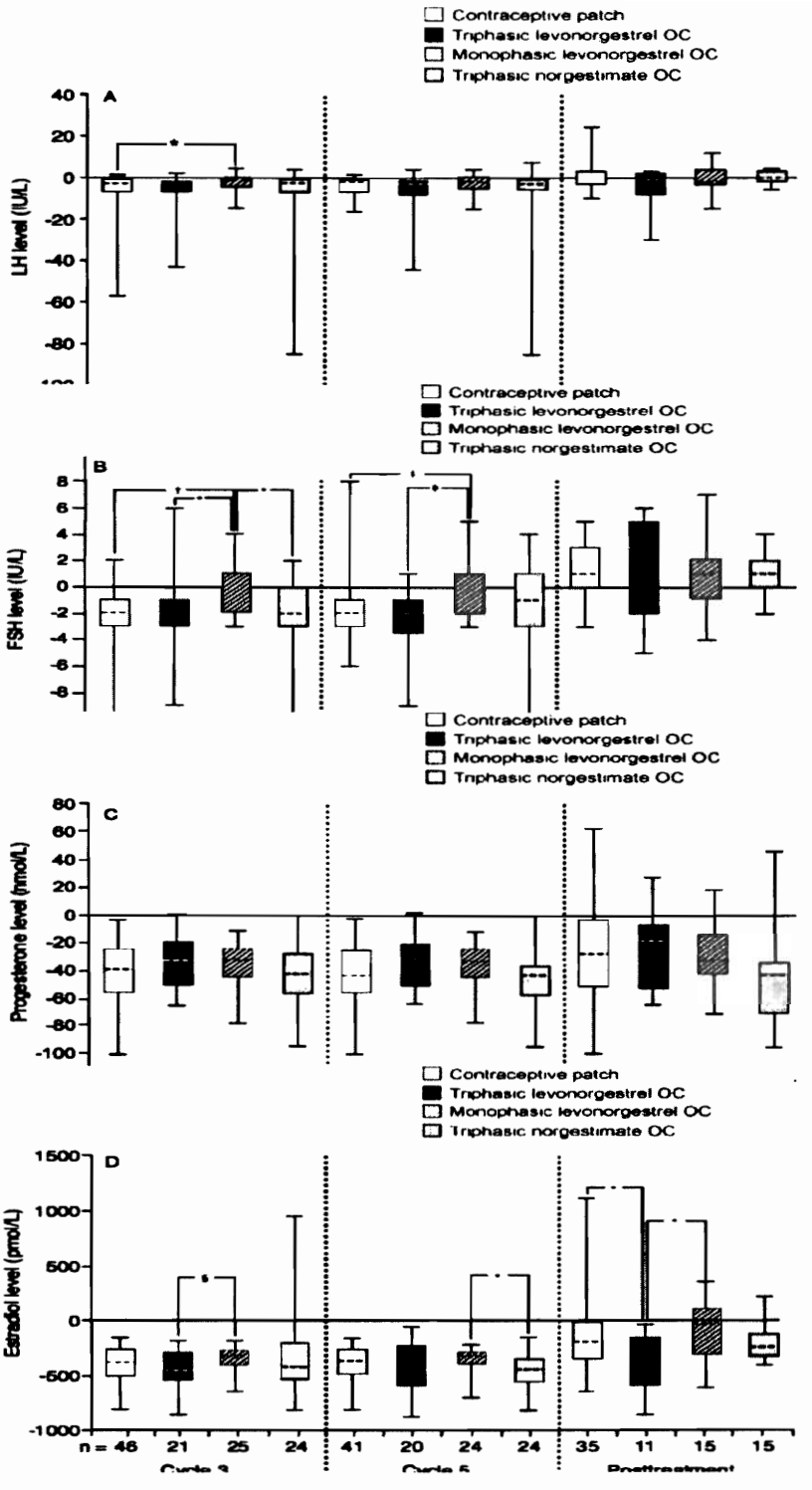

Figure A2. Mean changes from baseline to Cycle 3, from baseline to Cycle 5, and from baseline to 4 to 6 weeks post-treatment, in (a) luteinizing hormone (LH), (b) follicle-stimulating hormone (FSH), (c) progesterone, and (d) estradiol. The dashed lines indicate the mean values, with 50% of all observations included in the boxes; lines extending above and below the boxes indicate the maximum and minimum values, respectively. * $p < 0.05$; † $p < 0.001$; ‡ $p < 0.01$; § $p = 0.05$.

Patch adhesion

A total of 2.4% of patches (16 of 658) were replaced for complete detachment.

Safety

There were no unexpected adverse events. The most frequent adverse events were headache, intermenstrual bleeding, and abdominal pain. The adverse events profile was similar in all treatment groups, with the exception of mild-to-moderate application site reactions in the patch group. One subject in the contraceptive patch group discontinued because of a left paratubal cyst, and 1 subject in the monophasic LNG OC group discontinued due to a serious adverse event (torn ligament in right knee). No clinically meaningful changes from baseline in vital signs or physical or gynecologic examination findings were noted in any treatment group.

A5. Discussion

A primary objective of the present 5-cycle study was to compare the contraceptive patch and 3 commonly used and well-established OCs with regard to follicular size and the incidence of ovulation after normal dosing and after a 3-day dosing error in healthy, ovulatory women. Dosing errors were planned in Cycle 4. During the 10-day Cycle 4, OC users took the pill for 7 consecutive days followed by 3 pill-free days; contraceptive patch users either wore the patch for 7 consecutive days followed by 3 patch-free days (Group 2) or wore the patch for 10 consecutive days, 3 days longer than recommended (Group 1). Proper application of the patch is 3 consecutive weeks [21 days] followed by 1 patch-free week per cycle. The initiation of Cycle 5 immediately after the planned dosing error replicates the instructions for correction of dosing errors for each product.

The contraceptive patch groups had significantly lower follicular sizes than any of the OC regimens at Cycle 5 (following the dosing errors), and there were no significant differences in the follicular sizes between the 2 contraceptive patch groups. The incidence of ovulation in the comparator groups at Cycle 5 followed a similar pattern to that observed in Cycles 1 through 3. The contraceptive patch has been previously shown to maintain steroid serum concentrations within the reference

concentration ranges for 9 full days, which is 2 days beyond the recommended 7-day wear period.(7) The reference concentration ranges for norelgestromin (0.6–1.2 ng/mL) and EE (25–75 pg/mL) with contraceptive patch treatment are based on C_{avg} concentrations in 90% of individual subjects receiving NGM 250 μg/ EE 35 μg. Since the contraceptive patch has been shown to maintain steroid concentrations within the reference ranges for 9 days, it is not surprising that the 3-day dosing error in the present study had no impact on its ability to suppress ovarian activity.

The reason for similar follicular sizes after a 3-day period of "no patch" compared to a 3-day period of "prolonged patch wear" and for both contraceptive patch groups having significantly smaller follicular sizes than that following OC dosing errors may not be readily apparent. Three possible explanations are proposed. One possibility is that there is a "depot" effect in the skin, such that upon removal of the patch, plasma levels are maintained for a period afterward until the drug in the skin-contact-area is completely absorbed into the systemic circulation. Another possibility might be that the circulating levels of norelgestromin and EE are so high that there are measurable plasma levels for several days after patch removal. However, pharmacokinetic data do not support either of these possibilities. Pharmacokinetic data on norelgestromin and EE following removal of the contraceptive patch have been evaluated.(7,8) These studies showed that the plasma half-life of norelgestromin and EE following removal of the patch is similar to the half-life following oral dosing, providing evidence against any "depot" effect in the skin or of prolonged elevated levels. A third possible explanation is based upon the pharmacokinetic differences between transdermal and oral delivery. The cumulative daily delivery of norelgestromin and EE from the patch was designed to be similar to a NGM 250 μg/ EE 35 μg tablet (ORTHO-CYCLEN), similar to the triphasic OC in this study. Since the daily exposure to EE is similar for the contraceptive patch and the triphasic NGM OC, it is possible that continuous delivery of EE from the patch provides a greater effect than the peak-and-trough delivery of oral EE. The authors interpreted the findings to mean that for EE and its effect on suppression of follicular development (through suppression of FSH) as assessed by the follicular size, both the plasma profile and the delivered dose contribute significantly to the biologic effect.

The endpoint of ovulation in this study can be used to assess the contributions of both EE and the progestin component of the test regimens. Ovulation is dependent upon both FSH and LH, and while it is generally accepted that the EE component of a hormonal contraceptive is primarily responsible for FSH suppression, the progestin component is thought to be more important in suppressing LH. In this study, the contraceptive patch was associated with a significantly lower rate of ovulation than any of the OC regimens. One explanation might be that the progestin dose is greater with the patch; however, it has been shown that the daily exposure, or total bioavailability (area under the curve), for norelgestromin and EE with the contraceptive patch is similar to oral dosing with a NGM 250 μg/EE 35 μg tablet.(9) A more likely explanation is that the plasma concentration profile is more constant with the contraceptive patch than with OCs, resulting in a clearly different biologic effect.

The endocrine profile for the poststudy results shows that for all treatments the hypothalamic-pituitary-ovarian (HPO) axis activity was returning toward baseline in the 6-week period after the study. This is consistent with previous work showing a return to fertility with OC regimens.(10) From the results of this study, it is anticipated that following discontinuation of treatment with the contraceptive patch, return to fertility will be rapid, approximating that seen with OCs.

Only 2.4% of patches were replaced due to complete detachment in the study. In studies involving over 3000 contraceptive patch users, replacement rates for complete detachment have been reported to be < 2%.(11–14) All treatments were well tolerated in this study. Only 1 subject discontinued treatment with the contraceptive patch, and there were no drug-related serious adverse events. As expected, only subjects using the patch reported application site reactions, but none were treatment limiting and in all cases, were mild to moderate in severity.

In conclusion, follicular size and the incidence of ovulation were significantly lower after the use of the contraceptive patch compared with the use of OCs during cycles of correct contraceptive use and following 3 days of dosing errors.

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